Molecular mechanisms of bone invasion by oral squamous cell carcinoma

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Statement of Originality

I declare that the worked contained in this thesis was performed within Griffith University under the supervision of A/Prof. Nigel Morrison, Prof. Newell Johnson and Prof. Jin Gao.

This thesis represents the research performed for Doctor of Philosophy (PhD).

To the best of my knowledge all work performed by others has been referenced in this thesis.

This thesis has not been submitted for any other award or degree.

Jingjing Quan

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed)
Statement of Thesis Contribution

Chapter 1 of this thesis includes a co-authored published paper “Potential molecular targets for inhibiting bone invasion by oral squamous cell carcinoma: a review of mechanisms”, which has been published in the journal of “Cancer Metastasis Review”. I performed the literature review and drafted the manuscript. Prof. Newell Johnson and Prof. Jin Gao advised and performed the major revision of the manuscript structure and language. Guangbiao Zhou, Perter Parsons and Glen Boyle are co-authors who have advised on techniques for the overall study.

Chapter 3 of this thesis includes another co-authored published paper “Molecular pathways involved in crosstalk between cancer cells, osteoblasts and osteoclasts in the invasion of bone by oral squamous cell carcinoma”, which has been published in the journal of “Pathology”. I and Chuanxiang Zhou designed the experiments under the supervision of Prof. Jin Gao and Prof. Newell Johnson. Chuanxiang Zhou partially provided the data for gelatine zymography and western blotting, while I provided the other major data, drafted the full paper and added more data based on reviewers’ comments. Prof. Jin Gao and Prof. Newell Johnson performed the major revision of structure and language. Prof. Glenn Francis and Prof. Jane Dahlstrom supplied the tissue sections from OSCC patients, proofread the manuscript and supplied some language revisions.

Chapter 4 of this thesis has been written into a co-authored paper “Bone invasion by oral squamous cell carcinoma is mediated by epithelial-mesenchymal transition triggered by transforming growth factor-β1”, which has been submitted to the journal of “Clinical & Experimental Metastasis”. I designed the experiments, provided the
major data and drafted the manuscript. Moustafa Elhouiny provided the data from immunohistochemical staining. Prof. Newell Johnson and Prof. Jin Gao performed the major revision of structure and language.

The content of Chapter 5 in this thesis has been written into an abstract "Monocyte chemotactic protein-1: a potential target to inhibit the progression of bone invasion by oral squamous cell carcinoma", which has been accepted in the Cold Spring Harbor Asia Conference 2012 (Bone and Cartilage: from Development to Human Diseases). A related paper is being written now and will be submitted to a suitable journal shortly. I and A/Prof. Nigel Morison designed the experiments. I provided all data and drafted the manuscript. Prof. Jin Gao and Prof. Newell Johnson proofread the paper and performed the major revision of structure and language.
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Research Publications

Publications relevant to this thesis


3. Quan JJ, Elhouiny M, Johnson NW and Gao J. Bone invasion by oral squamous cell carcinoma is mediated by epithelial-mesenchymal transition triggered by transforming growth factor-β1 (submitted to Clinical & Experimental Metastasis, IF=3.524).


Other publications during PhD study


Conference oral presentations


Conference poster presentations

- Monocyte chemotactic protein-1: a potential target to inhibit the progression of bone invasion by oral squamous cell carcinoma, Cold Spring Harbor Asia Conference 2012 (Bone and Cartilage: from Development to Human Diseases), Suzhou, China, Poster Presentation.

- Cross-talk between osteoblasts and cancer cells in the invasion of oral squamous cell carcinoma, 2009 Gold Coast Health and Medical Research Conference, Australia, Poster Presentation.
Acknowledgement of published papers included in this thesis

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- Acknowledge all those who have contributed to the research, facilities or materials but who do not qualify as authors, such as research assistants, technical staff, and advisors on cultural or community knowledge. Obtain written consent to name individuals.
Included in this thesis are published papers in Chapters 1, 3 which are co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details for these papers are:

Chapter 1:


Chapter 3:


Appropriate acknowledgements of those who contributed to the research but did not qualify as co-authors are included in each published paper.

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Supervisor: Prof. Newell W.
Abbreviations

ALP  alkaline phosphatase
bp   base pair
BMC  bone marrow cell
BMP  bone morphology protein
BSA  bovine serum albumin
cDNA complementary DNA
CCL  chemokine (C-C motif) ligand
CK   cytokeratin
CM   conditioned medium
CSF  colony stimulating factor
DAPI 4',6-Diamidino-2-Phenylindole, Dihydrochloride
DMEM Dulbecco’s modified Eagle’s medium
DMSO dimethyl sulfoxide
DNA  deoxyribonucleic acid
E-cad E-cadherin
ECM extracellular matrix
EDTA ethylenediaminetetraacetic acid
EMT epithelial-mesenchymal transition
FACS fluorescence activation cell sorter
FBS  foetal bovine serum
FCS  foetal calf serum
GAPDH glyceraldehyde 3-phosphate dehydrogenase
H & E haematoxylin & eosin
HCl  hydrochloric acid
HRP horseradish peroxidase-conjugated
IHC  immunohistochemistry
IL   interleukin
Lab  laboratory
mRNA messenger RNA
MCP  monocyte chemoattractant protein
MEM  minimum essential media
MMP  
matix metalloproteinase

MTT  
methylthiazol tetrazolium

MT1-MMP  
membrane type 1 matrix metalloproteinase

N-cad  
N-caderin

NaOH  
sodium hydroxide

OB  
osteoblast

OD  
optical density

OPG  
osteoprotegerin

OSCC  
oral squamous cell carcinoma

PAGE  
polyacrylamide gel electrophoresis

PBS  
phosphate buffered saline

PBMC  
peripheral blood mononuclear cell

PCR  
polymerase chain reaction

PFA  
paraformaldehyde

PTHrP  
parathyroid hormone related peptide

rRNA  
ribosomal ribonucleic acid

RANK  
receptor activator of nuclear factor-κB

RANKL  
receptor activator of nuclear factor-κB ligand

RNA  
ribonucleic acid

RT  
reverse transcriptase

Runx  
runt-related transcription factor

SDS  
sodium dodecyl sulfate

TBS  
tris buffer saline

TGF  
transforming growth factor

TIMP  
tissue inhibitor of matrix metalloproteinase

TNF  
tumour necrosis factor

Twist  
twist-related protein

TRAP  
tartrate-resistant acid phosphatase

Tris  
tris(hydroxymethyl)aminomethane

VIM  
vimentin
Abstract

Invasion of bone is a common characteristic of oral squamous cell carcinoma (OSCC), which adversely affects on functionality and survival of patients. Recent studies suggest that osteoclasts, rather than OSCC cells, facilitate the entry of the tumour into bone and result in the progression of cancer within bone. Generation of osteoclasts is due to either an indirect effect involving stimulation of osteoblasts, or a direct effect initiated by OSCC cells. This study, consisting of three parts, aims to investigate the signalling pathways involved in the crosstalk between OSCC cells, osteoblasts and osteoclasts. Using research models both in vitro and in vivo, this study explores molecules with the potential to inhibit such bone invasion for the design of future biotherapies.

In part one, an indirect cell co-culture model was utilized to determine whether matrix metalloproteinases (MMPs), particularly MMP-2 & MMP-9, interacting with other molecules which regulate osteoblast differentiation and osteoclastogenesis, could play important roles in invasion of bone by OSCC. Conditioned medium (CM, supernatant) was collected from OSCC cell lines (SCC15 and SCC25), and from cultured osteoblasts (hFOB cell line and a primary culture, OB), which was used for indirect co-culture: OSCC cells were treated with CM from osteoblasts and vice versa. Results of gelatine zymography showed that zymogenic activities of both MMPs were increased in OSCC cells following culture with CM from hFOB. Western blotting showed the expression of Twist1 protein was increased, while Runx2 did not alter. The RANKL/OPG ratio, zymogen and protein expression of MMP-9 were increased in hFOB cells cultured with CM from OSCC cell lines, while zymogen expression of MMP-2 was decreased. Using real-time PCR, it was found that mRNA of these
molecules was consistent with the expression at protein levels. All targeted molecules were expressed in invading malignant keratinocytes, and all but OPG were expressed in osteoclasts of bone-invasive OSCCs obtained from clinical samples. This part demonstrates that crosstalk between different types of cell appears to exist in the invasion of bone by OSCC.

In part two, to determine which component of supernatant caused changes of gene expression in cell co-cultures, effects of transforming growth factor-β1 (TGF-β1) on OSCC cells and the way they interact with osteoclasts were examined. Three OSCC cell lines, SCC25, HN5, and Tca8113 were artificially induced to display epithelial-mesenchymal transition (EMT) by adding 5 ng/mL of TGF-β1 to culture media for 1-3 days. Results of methylthiazol tetrazolium (MTT) assay showed that TGF-β1 had no effect on proliferation of OSCC cells. Using immunocytochemistry (IHC), minimal staining of Vimentin (VIM) was found in SCC25 and HN5, while Tca8113 cells were strongly stained. In all cell lines assessed by real-time PCR, gelatine zymography and western blotting, expressions of EMT markers Twist1 and N-cadherin (N-cad) were up-regulated; Snail1 and E-cadherin (E-cad) down-regulated. Of factors associated with invasion, MMP-2 was unchanged and MMP-9 increased in SCC25 and Tca8113, while MMP-2 was increased and MMP-9 unchanged in HN5. For osteoclast-related molecules, both membrane type 1 (MT1)-MMP and RANKL were up-regulated, while OPG was down-regulated in all cells. CM obtained from OSCC cells which were pre-treated with TGF-β1 prolonged the survival of mature osteoclasts up to 4 days. This part suggests that partial EMT of OSCC cells, triggered by TGF-β1, may be associated with bone invasion by OSCC.
In part three, since monocyte chemotactic protein (MCP)-1 has been identified as a chemokine closely related with recruitment and activation of osteoclasts, we explored whether suppressed expression of MCP-1 can inhibit bone invasion by OSCC via its effects on osteoclasts. Results of IHC showed that strong staining of MCP-1 protein was observed in tumour cells and osteoclasts from 10 archival blocks of OSCC patients with bone invasion. Real-time PCR demonstrated that MCP-1 at the mRNA levels was highly expressed by all OSCC cell lines (SCC25, HN5, and Tca8113), but SCC25 cells had the highest expression level. A plasmid with the inhibitor of MCP-1 (7ND vector) was successfully transfected into SCC25 cells, as the stabilized SCC25 cells with 0.6 μg of 7ND vector (SCC25-7ND) were generated after G418 selection. Additionally, 10% CM of SCC25-7ND cells could efficiently inhibit the formation of human osteoclasts obtained from CD14+ monocyte subpopulation, compared with 10% CM of SCC25 cells. An animal model of bone invasion by OSCC was established by injecting tumour cells onto the surface of calvaria of nude mice. H&E staining showed that well-differentiated OSCC was formed in both groups of SCC25 and SCC25-7ND cell injections, with tumour cells invading the bone, while osteoclasts were found to locate in typical resorption lacunae. IHC suggested that MCP-1 was mainly localized in the cytoplasm and membrane of tumour cells in each group. TRAP staining indicated that there were no significant differences between two groups in cell numbers of osteoclasts recruited by tumour cells. Results in this part of the thesis demonstrate the relevance of MCP-1 with research on bone invasion by OSCC, and suggest the potential value of MCP-1 as a target to inhibit this common complication in clinical medicine.
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Appendix Publications from PhD

Publication 1-potential molecular targets for inhibiting bone invasion by oral squamous cell carcinoma: a review of mechanisms

Publication 2-molecular pathways involved in crosstalk between cancer cells, osteoblasts and osteoclasts in the invasion of bone by oral squamous cell carcinoma

Publication 3-correlation of the expression of human kallikrein-related peptidases 4 and 7 with the prognosis in oral squamous cell carcinoma

Publication 4-regulation of the stromal cell-derived factor-1alpha-CXCR4 axis in human dental pulp cells
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Chapter 1

Introduction and Literature Review
1.1 Oral squamous cell carcinoma

Globally, "oral cancer" is the eighth common cause of cancer-related death: more than 90% of oral malignancies are oral squamous cell carcinoma (OSCC).\textsuperscript{1-3} OSCC usually develops in sites of lip, gingiva, tongue, buccal mucosa or floor of mouth; therefore it severely affects patients' function of eating, swallowing and/or speech. Recent data indicate that 28,900 new patients of OSCC have been found and 7400 deaths could be attributed to it each year in USA alone.\textsuperscript{4} Furthermore, there is an increasing trend in oral cancer incidence of north and eastern europe.\textsuperscript{5} It is also reported that highest incidence rates of lip cancer are found in white population of Australia.\textsuperscript{6} During the period of 1983 to 1996, the incidence of lip cancer each year in Australian men increased from 8.3 to 9.2, while in women from 1.4 to 3.0 per 100000 people.\textsuperscript{1,6} Due to these large numbers of patients, OSCC has become an important public health issue, which draws considerable public attention in many countries.

No single factor leads to the development of cancer. OSCC is also caused by the combination of several intrinsic and extrinsic factors. The intrinsic factors include age, heritable disorders and genetic susceptibility.\textsuperscript{4} Nearly 90% of OSCC occur in old people with 65 years old as the average age; possibly age is a surrogate for accumulated exposure to carcinogens with their associated damage to normal cells.\textsuperscript{7} A few known heritable disorders have also been associated with increased incidence of oral cancer, for example, Fanconi Anemia (FA), which is an autosomal recessive syndrome, caused by defects in genes of recognizing and repairing DNA.\textsuperscript{8} It was demonstrated that people diagnosed with FA may have a 500 to 700 fold increased
risk of OSCC comparing with healthy people. Additionally, the risk of OSCC may depend on the genetic susceptibility. If an individual person has a positive family history (PFH) of oral cancer, he or she may have a higher genetic susceptibility to carcinogens, which suggests a greater risk of inherited cancer syndromes.

The established extrinsic factors include tobacco, alcohol and viruses. According to the latest reports, combined use of tobacco and alcohol accounts for almost three fourths of all oral cancers in USA. Both tobacco and alcohol contain several kinds of known carcinogens, which may disrupt normal cell proliferation or reduce the efficiency of immune system. Also mentioned are candidate viruses, such as human immunodeficiency virus (HIV), human papilloma virus (HPV), or human herpes virus HHV-8 (KSHV), which have been shown to disturb oncogenes and host cells’ function to increase the risks of OSCC. Additionally, some researches found that OSCC risk declined with increasing intake of vegetables and fruits. Meanwhile, chronic nutritional diseases, which result in the decreased body mass index (BMI), may contribute to the increased risks of OSCC, especially in developing countries. These researches suggest that a relationship may exist between nutrition and the incidence of oral cancer.

Despite improvements in therapies, the mortality of OSCC patients remains high. About half of the patients will be alive for only 5 years after the diagnosis. Early, patients are always painless and typical lesions are white, red, or a mixed patch in the oral mucosa. Later, superficial ulceration of the mucosa may appear. The typical features are central ulceration with rolled borders and induration. In more advanced stages, most OSCC spread via lymphatics to regional lymph nodes and distant blood-
borne metastases may also be found. The treatment of OSCC depends on the primary tumour site, tumour-nodes-metastases' (TNM) score, or the patient's overall health/presence of co-morbidities. Hotte et al reviewed all therapies of OSCC by using meta-analyses.\textsuperscript{13} They recommended surgery and/or fractionated radiotherapy rather than the chemotherapy for most oral sites. The use of altered fractionation can improve control of tumour and overall survival rates. But the benefits of chemotherapy are not evident and the impact of toxicity could affect physical function of patients. For metastatic sites, the combination of chemotherapy and radiation therapy appear superior to other combinations or a single treatment. Regardless of the treatment used, recoverable function of speech, mastication and swallowing of patients needs to be considered.

1.2 Bone invasion

Lesions occurring in the retromolar trigone, gingiva, hard palate, buccal mucosa extending to sulci, or tongue extending to floor of mouth may involve bone of the maxilla and/or the mandible. The prevalence of mandibular bone involvement by OSCC is reported to range from 12\% to 56\% of cases.\textsuperscript{14-16} Such patients commonly have severe dysfunction of speech, mastication and/or swallowing. Treatment and rehabilitation of cases involving bone is particularly challenging. Unfortunately, the cellular and molecular mechanisms of bone invasion by OSCC, and thus ways to prevent this, remain largely unknown. Understanding these mechanisms is essential in minimising spread and in planning surgical ablation, especially for guiding preservation surgery of the maxilla or mandible.
Pandey et al found that mandibular invasion was more common with tumours centred within 1 cm of the bone surface.\textsuperscript{17} Two patterns of invasion are recognized: an infiltrative and an erosive form. In the infiltrative pattern, malignant keratinocytes advance into cancellous spaces in small clusters or chords with little osteoclastic activity. This requires portals of entry through the cortex, such as fenestrations on a resorbed alveolar ridge, an incompletely healed extraction socket, or perhaps the periodontal ligament.\textsuperscript{18} In the erosive pattern, the neoplasm advances on a broad front, with an intervening connective tissue layer and active osteoclasts separating tumour from bone (Fig.1.1).\textsuperscript{19} Some researchers have found progression from the erosive to the infiltrative pattern, as the extent of bone involvement increases.\textsuperscript{20} The formation of these two patterns is likely to be influenced by anatomic details of area of exposed bone, in particular whether the advancing front of the neoplasm meets compact or cancellous bone, by intrinsic properties of malignant cells themselves, by properties of tumour stroma and factors unknown.\textsuperscript{21} The recent observation that, amongst 21 cases of gingival SCC, transforming growth factor-\(\beta\) (TGF-\(\beta\)) type I receptor was strongly expressed by tumour cells in all 14 cases of the infiltrative pattern, but in only 4 of 7 cases of erosive pattern, suggests locally active signalling pathways may alter cell behaviour to promote different patterns of bone invasion.\textsuperscript{22}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{patterns.png}
\caption{Patterns of mandibular bone invasion. (A) The infiltrative pattern. (B) The erosive pattern (Haematoxylin and eosin staining. Scale = 100 \(\mu\)m).}
\end{figure}
Despite improvements in the treatment of OSCC, high recurrence rates and shorter survival have been reported in patients with bony invasion. Shaw et al prospectively reviewed 100 consecutive mandibular resections and conducted a follow-up study of 65 months.\textsuperscript{19} They found that 62 mandibles were invaded by carcinoma and 21% of these patients had a recurrence within the study period. The 5-year disease-specific survival rate was 61% for patients with mandibular invasion, compared to 80% for those without. Recently, Ebrahimi and his colleagues published another retrospective review, with a larger sample of 498 patients, 102 of whom had histologically proven invasion of bone at initial surgery.\textsuperscript{23} After adjusting for other factors in a multivariate analysis, medullary invasion was the only independent predictor of reduced survival. These two substantial studies indicate the importance of detecting bone involvement in the planning of treatment and, if possible, finding ways to prevent it.

1.3 Molecular mechanisms

Although little is known as to whether OSCC cells have the ability to resorb bone themselves, it is clear that osteoclasts are much involved in this process.\textsuperscript{24-26} Osteoclasts differentiate at the bone surface and dissolve mineral components by an extracellular mechanism involving secretion of acid.\textsuperscript{27,28} The formation of osteoclasts is attributed to the stimulation of osteoblasts, which produce most of the bone constituents and differentiate from multipotent mesenchymal cells.\textsuperscript{29} The receptor activator of nuclear factor (NF)-$\kappa$B (RANK) ligand (RANKL), secreted by osteoblasts, binds to its receptor RANK on the surface of osteoclast precursors, subsequently leads to the formation and differentiation of mature osteoclasts.
Several signalling pathways initiated by products of the neoplastic keratinocytes themselves are reported to affect osteoclast function, the latter including proteases, cytokines, and growth factors. Bone invasion is a highly coordinated process, spatially and temporally regulated and can be described as having three phases: initial, resorption and final phases. Various molecules orchestrate these phases and appear to play distinct roles in each phase. In the initial phase, while osteoclasts have not yet been recruited, proteases help to degrade the extracellular matrix (ECM) of surrounding soft tissues, and facilitate the entry of malignant keratinocytes into the soft tissues or marrow spaces within the bone. The main phase of bone invasion is then the resorption phase, when osteoclasts take the main role to resorb the calcified components of bone. At this stage, cytokines generated from tumour cells directly or indirectly induce the formation of osteoclasts. Following osteoclast-mediated resorption, additional growth factors are liberated and these promote the growth of neoplastic cells themselves, thus driving a vicious cycle to accelerate the process of bone invasion.

\subsection*{1.3.1 Proteases-initial phase}

Bone tissue consists of a number of distinct cell types and the bone matrix, which is largely mineralized. In the bone matrix, crystals of hydroxyapatite exist in association with both collagenous and noncollagenous proteins: the latter including osteocalcin, osteopontin, osteonectin and calbindin.\textsuperscript{30,31} Osteocytes are incorporated within bone, together with other cell types, such as osteoprogenitor cells, osteoblasts, osteoclasts, marrow fibroblasts, and undifferentiated cells, all of which participate in the process of continuous bone remodelling.\textsuperscript{30,31} Malignant keratinocytes produce a range of
enzymes capable of destroying bone matrix directly, enhancing their migration into the adjacent soft tissue spaces. Moreover, these proteolytic enzymes act on bone cells, especially osteoclast precursors, to promote their differentiation and maturation, thus causing further bone destruction. Two major groups of proteases play a part in bone invasion of OSCC: matrix metalloproteinases and cathepsins.

1.3.1.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family composed of at least 26 members. These are the main proteolytic enzymes responsible for digestion of fibrillar and non-fibrillar collagens, elastin, gelatine and proteoglycans. Studies of the function of MMPs in vivo have also revealed that these proteases cleave and activate other extracellular protein substrates: indeed MMPs are involved in a variety of homeostatic functions including angiogenesis, wound healing and stimulation of immune responses.

Erdem et al showed that MMP-1 and MMP-9 were highly expressed by BHY cells, a cell line derived from a SCC of the lower alveolus which had deeply invaded the mandible. Using immunohistochemistry (IHC) and flow cytometry, they also detected high levels of expression of extracellular matrix metalloproteinase inducer (EMMPRIN) in these cells. Another research group found that tumour tissues from 9 out of 24 cases of buccal SCC with mandibular invasion, were stained strongly for active MMP-7, compared to 15 cases without invasion of the adjacent bone. This is the first study demonstrating that MMP-7 is correlated with mandibular invasion, a significant observation which is consistent with the demonstration by others that,
secretion of MMP-7 by prostate and breast cancer cells is capable of cleaving RANKL to an active soluble form, which then induce formation of osteoclasts and bone resorption.\textsuperscript{37,38}

In the preliminary study of our group, we verified that molecules of MMP-2 and MMP-9 were indeed present \textit{in vivo}, using IHC on tissue sections of OSCC invading bone: MMP-2 was weakly expressed while MMP-9 was strongly expressed within the cytoplasm of invading malignant keratinocytes (Fig.1.2). This suggests that MMP-2 and MMP-9 may have important roles in the stimulation of bone invasion by OSCC, which was further studied in Chapter 3.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image}
\caption{Expression of MMP-2 and MMP-9 in immunohistochemical sections from OSCC with bone invasion. (A) H&E staining demonstrates an infiltrative pattern of bone invasion. (B) MMP-2 was weakly stained in the cytoplasm of invading malignant keratinocytes. (C) MMP-9 was strongly expressed.}
\end{figure}
1.3.1.2 Cathepsins

Cathepsins are members of the endosomal/lysosomal protease family, which are active at low pH. They play an important role in bone degradation by osteoclasts. The cathepsin family has more than 21 members, divided into four subgroups: cysteine proteases, aspartic proteases, serine proteases and tripeptidyl peptidase I.\textsuperscript{39} Most, including cathepsins B and L, belong to the cysteine protease group; whereas cathepsins D and E are aspartic proteases.

Evidence for the role of cathepsins again comes from human studies, from animal experiments and from work with cell lines \textit{in vitro}. Kawamata et al detected that BHY cells secreted a large amount of procathepsin L.\textsuperscript{40} After injection into the masseter muscle of nude mice, BHY cells developed into highly differentiated squamous cell carcinomas (SCCs) which invaded the mandible, indicating that cathepsin L might help to degrade the bone matrix. This orthotopic animal model, in which tumour cells are injected adjacent to the mandible itself, has been frequently used in early studies of bone invasion by OSCC.\textsuperscript{41} However, based on our laboratory experience, injecting through the masseter muscle can cause some discomfort and feeding difficulties for the animals, which may result in unexpected death during the middle of tumour development. Therefore, this animal model needs to be improved. Another study showed that cathepsins B and D were strongly expressed in all 78 human SCCs of tongue, gingiva or floor of mouth.\textsuperscript{42} Using one way ANOVA, the labelling indices of both cathepsins had a significantly close correlation with degree of bone invasion. Furthermore, in this case series, patients with high serum levels of cathepsins B and D
had shorter survival time, suggesting these molecules may be of value as prognostic markers.

1.3.2 Cytokines—resorption phase

Osteoclasts are specific polykaryons, derived from monocyte/macrophage precursor cells. It is known that two hematopoietic factors are necessary for osteoclastogenesis, RANKL and colony-stimulating factor-1 (CSF-1). In addition, other systemic hormones or cytokines regulate function of osteoclasts. In invasion of bone by OSCC, tumour-derived cytokines such as tumour necrosis factor-α (TNF-α) can directly or indirectly induce the formation of osteoclasts, by stimulating RANKL or CSF-1 expression. Furthermore, interleukins (IL), a family of pleiotropic cytokines secreted by osteoblasts, osteoclasts, and stromal cells, have members which are important regulators of bone metabolism, especially IL-6. Another important molecule of bone homeostasis, parathyroid hormone related peptide (PTHrP), which functions to prevent apoptosis of osteoblasts and to recruit osteoclasts, has been shown to be up-regulated in clinical tissues of OSCC.

1.3.2.1 TNF-related proteins

Recent studies have found that a family of TNF receptor (TNFR)/TNF-like proteins: osteoprotegerin (OPG), RANK and RANKL, regulate osteoclast differentiation. While RANKL is an inducer of bone resorption through its interaction with RANK, OPG is a soluble decoy receptor which acts as an inhibitor.
Ishikuro et al found that, of all 97 patients with gingival SCC, 45 cases were of the infiltrative type while the other 52 cases were of the erosive type. In all cases, some fibrous connective tissues intervened between tumour and bone cells. However, there were more fibroblasts in the infiltrative type than the erosive type. To further explore the roles of fibrous stroma, IHC was performed: this revealed that RANKL staining was mostly found in those fibroblasts in close apposition to osteoclasts. The authors also detected that, BHY cells stimulated expression of RANKL mRNA in mouse osteoblasts in cell co-culture experiments. Therefore, these results suggest that neoplastic keratinocytes of gingival SCC may affect the fibrous stroma, which then plays promoting roles in osteoclastic bone resorption.

Tada et al showed that TNFα released from OSCC cells partially contributes to the generation of osteoclasts, and tumour-induced suppression of OPG may be critical. When adding OPG into a co-culture between OSCC cells, mouse primary osteoblasts (POBs) and bone marrow cells (BMCs), osteoclast differentiation was significantly inhibited. This is consistent with the observation that, OSCC cells did not enhance osteoclastogenesis in co-culture between BMCs and POBs of OPG-deficient mice. These authors also examined the invaded mandibular lesions of 5 patients with OSCC, and found by IHC that, a low expression of OPG in osteoblasts and other stromal cells, in comparison to normal bone from the same patients, which further confirmed their observation in vitro. This study is innovative in its research design, for it compared two types of established OSCC cell lines with bone invasive properties.

1.3.2.2 Interleukins
Osteoclasts and immune cells share many regulatory molecules, perhaps because many of the latter develop in the same microenvironment as osteoclasts: namely in bone marrow.\(^{49}\) The finding that cultured human peripheral blood leukocytes can resorb bone is the evidence of a relationship between immune response and bone metabolism.\(^{50}\) The major mediator is now known to be interleukin-1β (IL-1β),\(^{51}\) but IL-6, IL-11 and IL-15 also stimulate bone resorption.\(^{52}\)

Okamoto et al found that BHY cells, which have the property of bone invasion, produced large amounts of IL-6, and CM from BHY cells markedly promoted pit formation by osteoclasts on mineralised substrates.\(^{53}\) These authors also found that invasion of the mandible by BHY cells in nude mice was efficiently inhibited, when adding anti-IL-6 antibody to the cells before they were injected into the masseter muscle. IL-6 thus appears to be a strong mediator or enhancer of bone invasion by oral cancer.

Another study investigated 38 cases of SCC of the lower gingiva, which were histopathologically classified into invasive and non-invasive groups.\(^{54}\) IHC showed that tumour cells were markedly more reactive for IL-6 and IL-11 in the invasive group, whereas expression of IL-1α, IL-1β and IL-18 were not significantly different between these two groups. However, Van Cann et al found that expression of IL-6 and IL-11 were not significantly different between biopsy specimens of OSCC with or without medullary invasion, and suggested that immunohistochemical detection of these markers might not be an appropriate method to predict mandibular invasion.\(^{55}\) Although results of these two studies are in contrast with each other, and the reasons for these differences may be caused by different procedure methods, these studies
indicate that bone invasion by OSCC is a complicated progression with many molecules involved.

1.3.2.3 Parathyroid hormone-related peptide

Parathyroid hormone related peptide (PTHrP), not parathyroid hormone (PTH) itself, was initially reported as a major factor related to malignancy-associated hypercalcaemia (MAH). PTHrP binds to its receptor, stimulates osteoclast activities and mediates bone destruction. It can also decrease expression of alkaline phosphatase (ALP) by osteoblasts, and their production of type I collagen, osteopontin, osteonectin, and osteocalcin.  

Deyama et al found that BHY cells naturally expressed high levels of PTHrP mRNA. Furthermore, PTHrP mRNA could be detected from tumour tissues in 7 of 11 patients with lower alveolus and gingival carcinoma, which showed an infiltrative pattern of bone involvement. The same group further demonstrated in two OSCC cell lines derived from cases with bone invasion, that PTHrP mRNA could be detected in abundance and significantly induced by epidermal growth factor (EGF), indicating PTHrP may contribute to signalling downstream of EGFR. 

Takayama et al showed that knock-down of PTHrP in OSCC cells caused dramatic reduction of osteoclast numbers in cell co-culture models. Addition of TGF-β induced expression of PTHrP, and generated epithelial-mesenchymal transition (EMT) of oral cancer cells, changes which might be expected to enhance bone invasion if these pathways were active in vivo. Consistent with this, they observed that
tissue sections of carcinomas with mandibular invasion showed large numbers of osteoclasts, and strong expression of PTHrP in tumour cells. E-cadherin staining of tumour cells was weaker at the bone resorption front than more central in the tumour mass, consistent with EMT-like changes. Taken together, these data suggest that PTHrP expression might have value as a predictor of bone involvement by OSCC.

1.3.2.4 Chemokines

Chemokines are small molecule cytokines, that exert their effects by binding to G protein coupled 7-span trans-membrane receptors.\textsuperscript{60} Some of these chemo-attractants are present in bone matrix and are thought to play a pivotal role in osteoclast activation. Furthermore, chemokines secreted by tumour cells themselves are also known to regulate recruitment and mobilization of osteoclasts.\textsuperscript{61}

Tang et al reported that the chemokine stromal cell-derived factor-1\(\alpha\) (SDF-1\(\alpha\)), also named CXCL12, increased IL-6 expression in cultured OSCC cells, which further promoted osteoclast differentiation in co-culture models \textit{in vitro}.\textsuperscript{62} rhSDF-1\(\alpha\) increased the expression of its receptor CXCR4 in these OSCC cells, and CXCR4 inhibition abolished SDF-1\(\alpha\)-induced IL-6 production. These authors further demonstrated that pathways of extracellular signal-regulated kinases (ERKs) and NF-\(\kappa\)B were involved in this transcriptional regulation.

Pandruvada et al found that both the chemokine ligand CXCL13 and its receptor CXCR5 were present in several OSCC cell lines, namely SCC1, SCC12 and SCC14a.\textsuperscript{63} They demonstrated that recombinant human CXCL13 (rhCXCL13) could
enhance the chemotaxis of peripheral blood monocytes in a dose-dependent manner, which would be expected to facilitate recruitment of osteoclasts. They injected OSCC cells onto the surface of calvaria in nude mice, where tumours developed in 4-5 weeks; histochemical staining then confirmed expressions of CXCL13 and CXCR5, implying that CXCL13 may be considered as a prognostic marker for bone invasion by OSCC. This "calvaria-injection" animal model represents a superior model for OSCC development and its progression of bone invasion. It has the distinct advantage of causing animals limited stress as there is no interference with mastication, which is a risk when using oral or submasseteric sites. This animal model was also used in the animal work of Chapter 5.

1.3.3 Growth factors-final phase

In addition to these cytokines, a variety of growth factors affect function of osteoblasts and osteoclasts during bone remodelling. Bone degradation in the progression of OSCC allows release of growth factors from reservoirs within mineralized matrix, such as epidermal growth factor (EGF), transforming growth factor (TGF), or connective tissue growth factor (CTGF), which may act as local regulators of tumour survival and bone resorption.64

Huang et al found that the anti-EGF receptor (EGFR) antibody C225 could decrease migration abilities of SCC-1 cells.65 Using a murine xenograft model, they detected that locoregional invasion of SCC-1 cells into blood vessels, muscle and bone was efficiently inhibited in C225-treated mice. These data provide evidence that blockade
of EGFR may suppress tumour-induced bony invasion as well as proliferation of the malignant keratinocytes themselves.

Prime et al examined the malignant behaviour of several OSCC cell lines by transplanting them to the floor of mouth in athymic mice.\textsuperscript{66} They found that cell lines of BICR31 and BICR56, which were resistant to TGF-β1-induced growth inhibition, formed more primary tumours than those cells that were growth inhibited by TGF-β1. Furthermore, invasion of the mandible was commonly found: a high incidence of 30% and 44% respectively. These results indicate that OSCC cells, unresponsive to TGF-β1 may behave more aggressively.

Shimo et al investigated the role of CTGF in destruction of the mandible. IHC from 20 cases of mandibular SCC showed the presence of CTGF in osteoclasts and tumour cells.\textsuperscript{67} In cultures of mouse bone marrow, rhCTGF stimulated the formation of tartrate resistant acid phosphatase (TRAP)-positive osteoclast-like cells. These results suggest that CTGF might be regarded as a diagnostic marker for the production of osteolytic lesions.
**Figure 1.3.** Various molecules expressed from OSCC cells lead to the bone invasion through osteoclastogenesis. (A) In the initial phase, proteases help to degrade the ECM and facilitate OSCC cells entering into the bone tissue. (B) In the resorption phase, these cytokines generated from tumour cells directly or indirectly induce the formation of osteoclasts. (C) In the final phase, growth factors are liberated and promote the growth of OSCC cells, which drive the vicious cycle to accelerate the bone invasion process.
1.4 Epithelial-mesenchymal transition

Studies of the mechanisms by which distant bone metastases develop, which occur particularly with tumours of breast, prostate, thyroid, kidney and lung, encourage us to rethink about the mechanisms of bone invasion by OSCC. Bone metastasis is a complex procedure which includes several steps. Cancer cells firstly need to separate from the primary sites, and then invade the adjacent blood vessels. Once in the blood, tumour cells are attracted to the target organs. Stephen Paget proposed the 'seed and soil' hypothesis, who indicated that a metastasis depends on the characteristics of the tumour cells and the microenvironment of targeted organ. In other words, a secondary tumour is established only if the seed can grow in the soil.

In order to exit from a primary tumour, recent findings demonstrate that cancer cells acquire the mesenchymal phenotype and become more active and migratory after an epithelial-mesenchymal transition (EMT), which leads to invasion into stroma, intravasation, dissemination and colonization of distant sites. The hallmark of EMT is the disintegration of cell-cell junctions particularly adherins junctions, and this dissociation is regulated by several transcription factors like the zinc-finger proteins of Snail and Slug. Accumulating evidence show that a number of growth factors such as TGF-β or EGF are potent effectors of EMT and are released from the microenvironment during the vicious cycle. Moreover, EMT programme has now been found to promote the self-renewal capability of carcinoma cells. For example, TGF-β, which induces E-cadherin repressors, has been reported in the generation of cancer cells with higher stem cell properties that are capable of tumour initiation and regeneration.
EMT transformation is reported in the progression of bone invasion by OSCC. Davies et al showed that transfection of TGF-β1 caused changes in cell morphology from polygonal to spindle shape in a rat keratinocyte cell line. These spindle carcinoma cells formed more tumours with higher incidence of local bone resorption. Our group found that long term treatment (6 days) with TGF-β1 (5 ng/mL) triggered the EMT changes of OSCC cells in vitro, which was associated with the increased expressions of MMP-2 and MMP-9. However, a short period of treatment (3 days) only induced partial EMT changes, since full morphological changes were not observed. But these changes could prolong the survival of mature osteoclasts and relate with bone invasion, which was further discussed in Chapter 4. Such findings generate a question of whether changes in the cellular morphology are necessary for acquiring osteomimetic characteristics, since some of bone metastatic cancer cells are demonstrated to undertake phenotype changes and become fibroblast-like cells, with further expression of osteogenic molecules. It is known that primary or metastatic cancers do not exist as isolated cells but, rather, they closely interact with a variety of stroma cell types. It is hard to speculate whether these tumour cells have gone through phenotypic changes, since the EMT program is transitory, in which there are technical difficulties to capture the whole process.

Cancer stem cells (CSCs), also known as tumour initiating cells, are a small subpopulation within a neoplasm, and have exhibited a higher capacity of promoting tumour growth, maintenance, metastatic progression, and contributing to radioresistance and chemoresistance. Malignant keratinocytes with EMT morphology and properties are typically found at the invasive front of primary tumours, and as part of the invasion of bone by OSCC. Could these be CSCs?
Could growth factors, especially TGF-β1, enrich the population of CSCs in OSCC with high invasive abilities? A potential model to examine these hypotheses may be the cell lines BICR31 and BICR56, which were resistant to TGF-β1-induced growth inhibition, and formed more primary tumours with high properties of mandibular invasion.\textsuperscript{66} However, more evidence is needed to prove these hypotheses.

1.5 Clinical implications

Oral squamous cell carcinomas which have invaded bone have a high recurrence rate, which dramatically impacts patients' recovery and quality of life. Before starting the treatment, a key point of diagnosis is to evaluate the presence and extent of tumour invasion. At present, preoperative assessments usually consist of clinical examination, conventional radiography by orthopantomogram (OPG) and periapical radiographs, and if available, computerised tomography (CT), radionuclide bone scanning and magnetic resonance imaging (MRI).\textsuperscript{85} Other more sophisticated diagnostic tools such as multi-detector row CT (MDCT) or positron emission tomography/CT (PET/CT) have been shown to have a higher sensitivity in the assessment of bone invasion, but are not for routine use in most institutions.\textsuperscript{86,87} An optimal combination of preoperative examination methods to predict bone invasion is necessary for the planning of definitive therapy for these patients.\textsuperscript{88}

The management of bone invasion in such patients remains controversial. Treatment choices include surgery, radiotherapy, chemotherapy and combinations thereof.\textsuperscript{89} Most clinicians choose surgery as the primary therapy, with postoperative radiation or chemoradiation depending on the tumour extent and stage. When the maxilla or
mandible is involved, marginal or segmental resection is needed and the primary aim of resection is to obtain clear surgical margins. However, these resections may cause decreased function and cosmetic deformity. Therefore, to maintain quality postoperative function, restoration of bone continuity is required, together with soft-tissue flaps to reconstruct the cosmetic defect. The post-surgical combined radiotherapy and chemotherapy are also helpful and reported to increase the overall survival rate. For example, Do et al showed that similar outcome would be achieved, between patients with bone or cartilage invasion treated with upfront chemoradiotherapy (CRT) or resection followed by CRT. However, the protocols of these postoperative therapies vary in different clinical centres and can only be applied in a subpopulation of OSCC patients at certain disease stage. These supplemental therapies are also associated with a substantial increase in adverse effects, including severe mucositis or osteoradionecrosis, which would severely affect patients' recovery and function. In a word, since each treatment has advantages and disadvantages, the personalized or customized treatment plan is necessary for fully functional recovery of these OSCC patients with bone invasion.

Bisphosphonates (BPs) are stable analogues of pyrophosphate, which are effective inhibitors of bone resorption. Thus, BPs are recognized as a treatment option for bone diseases with excessive activities of osteoclasts, such as osteoporosis, Paget's disease of bone, and skeletal complications induced by malignancy.

BPs inhibit bone resorption by adsorption to mineral surfaces in bone, where they interfere with osteoclasts. BPs can affect osteoclasts by inhibiting recruitment and differentiation of osteoclasts, inhibiting osteoclastic activities and inducing apoptosis...
of osteoclasts. They have been used intensively in oncology to minimize the complications of metastatic bone disease. For example, BPs have been reported to reduce skeletal morbidity with multiple myeloma by 30% to 50%. For bone invasion by OSCC, a recent report by Martin et al demonstrated zoledronic acid (ZOL), Nitrogen-containing BPs, inhibited OSCC-induced osteolysis in a murine xenograft model. They suggested ZOL to be valuable as an adjuvant therapy for OSCC patients with bone invasion.

A recognized side effect of BPs is the development of osteonecrosis of the jaws (ONJ), which is characterized by exposure of bone which fails to heal over prolonged periods, eg 6 to 8 weeks. Patients with OSCC involving bone may be at increased risk for ONJ. Clinicians should carefully consider relative risks of ONJ against the possibility or reduced spread of the neoplasms, which are dependent on the precise type of BPs prescribed, the dose and infusion time. Other risk factors for ONJ include radiation therapy, dental trauma and odontogenic and periodontal infections. Preventative dental care is recommended for all patients.

1.6 Biotherapy

With breakthroughs in biological target therapies, the treatment options for patients have been greatly expanded. Some of the mediators discussed above have already been used as targets, such as the human monoclonal antibody targeting RANKL, Denosumab, which has been globally used and achieved outstanding effects in the phase 3 clinical trials of patients with prostate cancer. RANKL inhibition by using recombinant OPG was also examined in patients with breast cancer or multiple...
myeloma with osteoclastic bone lesions. Shin et al found that treatment with OPG decreased bone invasion of human OSCC B88 cells, after being injected into the masseter region of nude mice. These examples have reminded researchers of the importance of bone invasion by OSCC. Efficient markers to quickly predict the presence of bone invasion, and effective targets to successfully treat the patients are needed for oral oncologists. As for the bone invasion, whilst the pathways are complex and interactive, molecular markers such as those described above would play a role in detecting the presence and extent of bone involvement. Furthermore, blockade of a variety of pathways including the initiation of cancer stem cells and BMT may open possibilities for future biotherapies with improved clinical outcomes.
1.7 Hypotheses and specific aims

Hypotheses

MMPs, along with molecules known to affect osteoblast differentiation and osteoclastogenesis, play key roles in the invasion of bone by OSCC.

Soluble molecules degraded from the bone matrix such as TGF-β1, mediates bone invasion of OSCC by altering the behaviour of both OSCC cells and osteoclasts. Inhibiting the expression of chemokine of MCP-1 in OSCC cells efficiently affect osteoclast formation, and disrupt the progression of bone invasion by OSCC.

Specific aims

To study whether MMPs, specifically MMP-2 & MMP-9, interacting with molecules of Twist1, Runx2, RANKL and OPG, could play important roles in the invasion of bone by OSCC.

To determine whether soluble molecules of CM can induce changes of gene expression in the indirect cell co-culture model, and examine effects of TGF-β1 on OSCC cells via EMT and the way they interact with osteoclasts.

To explore whether suppressed expression of MCP-1 in OSCC cells can be used as an efficient way to inhibit bone invasion by OSCC via targeting on the formation of osteoclasts.
Chapter 2

Materials and Methods
2.1 Materials

2.1.1 Reagents for cell culture

0.22 μm filter from Merck
0.25% trypsin-EDTA (1 x) with phenol red from Life Technologies
3.5 mL transfer pipets from Becton Dickinson
6 well tissue culture plates from Thermo Fisher Scientific
7ND vector from Kyushu University
10 mL non pyrogenic serological pipets (with plug) from Corning
15 mL centrifuge tubes from Corning
24 well tissue culture plates from Thermo Fisher Scientific
25 cm² tissue culture flasks from Thermo Fisher Scientific
25 mL non pyrogenic serological pipets (with plug) from Corning
50 mL centrifuge tubes from Corning
75 cm² tissue culture flasks from Thermo Fisher Scientific
96 well tissue culture plates from Thermo Fisher Scientific
Acetone from Merck
Acetic acid glacial from Merck
BD vacutainer cell preparation tubes (CPT) with sodium citrate from BD
Biomek plate reader from Beckman
Bovine serum albumin (BSA) from Sigma-Aldrich
Cell culture incubator from Shel Lab
Centrifuge from Eppendorf
CD14 microbeads of human from MACS
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) from Life Technologies
Dimethyl sulfoxide (DMSO) from Sigma
Dulbecco's modified Eagle's medium (DMEM) from Life Technologies
Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12) from Life Technologies
Ethylenediaminetetraacetic acid (EDTA) from Sigma-Aldrich
Fast red violet LB salt from Sigma-Aldrich
Foetal bovine serum (FBS) from Life Technologies
Foetal calf serum (FCS) from Life Technologies
FuGENE HD from Promega
Geneticin selective antibiotic (G418 Sulfate) from Life Technologies
Hemacytometer from Hauser Scientific
Hydrochloric acid (HCl) from Merck
Magnetic cell separator from MACS
Methylthiazol tetrazolium (MTT) from Life Technologies
Microscope from Olympus
Minimum essential media (MEM) alpha modification from Hyclone
Naphthol AS-MX phosphate from Sigma-Aldrich
Nikon OXM1200 digital camera from Nikon
Nikon TP2 digital camera from Nikon
Opti-MEM reduced serum media from Life Technologies
Penicillin-Streptomycin from Life Technologies
Phosphate buffered saline (PBS) tablets from Life Technologies
Pipette control from Bio-Rad
Recombinant human TGF-β1 From R&D
Recombinant human CSF1 from Pepro Tech
Recombinant human sRANK ligand from Pepro Tech
Recombinant mouse RANKL from R&D
Rhodamine phallodin from Life Technologies
Silver nitrate from Sigma-Aldrich
Sodium acetate (trihydrate) from Sigma-Aldrich
Sodium hydroxide (NaOH) from Merck
Sodium L-tartrate disbasic dehydrate from Sigma-Aldrich
Sodium thiosulfate from Sigma-Aldrich
TRAP staining kit from Nanjing Jiancheng
Triton X-100 from Chem-Supply
Ultra centrifuge from Beckman

2.1.2 Reagents for RNA isolation, cDNA synthesis and realtime-PCR

0.2 mL flat cap PCR tubes from Molecular BioProducts
0.2 mL strip tubes from Molecular BioProducts
1 mL Nichipet from In Vitro
1 mL pipet tips from Molecular BioProducts
1.5 mL microfuge tubes from Eppendorf
10 μL Nichipet from In Vitro
10 μL pipet tips from Molecular BioProducts
100 bp DNA ladder from Thermo Fisher Scientific
200 μL Nichipet from In Vitro
200 μL pipet tips from Molecular BioProducts
DNA grade agarose from Bio-Rad
DNA loading dye (6 x) from Thermo Fisher Scientific
EXPRESS SYBR GreenER qPCR SuperMixes from Life Technologies
iCycler iQ real-time detection system software version 3.0 from Bio-Rad
iCycler iQ system from Bio-Rad
iQ SYBR Green Supermix from Bio-Rad
iScript cDNA Synthesis Kit from Bio-Rad
Magnesium chloride (MgCl₂) from Promega
Microfuge-Centrifuge 5402 from Eppendorf
NanoDrop 1000 from Thermo Fisher Scientific
OligoDT from Promega
PowerPac 200 power supply from Bio-Rad
PureLink RNA Mini Kit from Life Technologies
Random primers from Promega
Reverse transcriptase (RT) from Promega
RNA sample loading buffer without ethidium bromide from Sigma-Aldrich
RT reaction buffer (5 x) from Promega
Set of 100 mM dATP, dCTP, dGTP, Dttp (dNTP) from Promega
Sub-Cell GT Cell from Bio-Rad
Sub-Cell GT UV-Transparent Mini-Gel Tray from Bio-Rad
SYBR Safe DNA gel stain from Life Technologies
Tris acetate-EDTA buffer (10 x) from Sigma-Aldrich
TRIzol from Life Technologies
UltraPure DNase/RNase-Free distilled water from Life Technologies
2.1.3 Reagents for gelatine zymography and western blotting

10% Ready gel zymogram precast gels from Bio-Rad
0.22 μm PVDF membranes from Merck
40% Acrylamide/Bis solution (37.5:1) from Bio-Rad
2-Mercaptoethanol from Bio-Rad
24 cm cell scrapers from TPP
Ammonium persulfate (APS) from Amresco Inc
BCA protein assay kit from Thermo Fisher Scientific
Bromophenol blue from Sigma-Aldrich
Chemiluminescence (ECL) western blotting substrate from Thermo Fisher Scientific
Comassie brilliant blue R-250 from Bio-Rad
Digital camera from Panasonic
GelCode blue safe protein stain from Thermo Fisher Scientific
Glycerol from Chem-Supply
Glycine from Merck
Halt protease inhibitor cocktail (100 x) from Thermo Fisher Scientific
Laemmli sample buffer from Bio-Rad
Methanol absolute from Merck
Mini-PROTEAN tetra cell from Bio-Rad
Mini Trans-Blot module from Bio-Rad
N,N,N',N'-Tetramethylethylenediamine (TEMED) from Sigma-Aldrich
PowerPac 300 power supply from Bio-Rad
Prestained protein ladder from Thermo Fisher Scientific
RIPA buffer (1 x) from Thermo Fisher Scientific
SDS from AppliChem

Skim milk powder from Diploma

SuperSignal WestPico chemiluminescent substrate from Thermo Fisher Scientific

Tris from AppliChem

Tris/Tricine/SDS Buffer (10 x) from Bio-Rad

Tween from Merck

VersaDoc-MP Imaging Systems from Bio-Rad

Zymogram development buffer from Bio-Rad

Zymogram renaturation buffer from Bio-Rad

2.1.4 Reagents for tissue sample preparation and staining of paraffin sections

1 mL syringes from BD

26g needles from TERUMO

FACScan flow cytometer from BD

CellQuest software from BD

Citric acid from Sigma-Aldrich

Cytoseal 60 mounting medium from Thermo Fisher Scientific

Dental probe from Gold Coast Hospital

DPX mounting medium from Leica

EnVision Detection Systems, Peroxidase/DAB, Rabbit/Mouse from Dako

Eosin solution alcoholic from Sigma-Aldrich

Ethylenediaminetetraacetic acid (EDTA) disodium salt from Sigma-Aldrich

Ethanol absolute from Merck

Fc receptor blocker from Innovex Biosciences
Formaldehyde solution 37/10 from Chem-Supply

Hematoxylin Solution, Harris Modified from Sigma-Aldrich

Leica RM 2235 rotary microtome from Leica

Light microscopy microscope from Olympus

Liquid-repellent slide marker pen from Daido Sangyo

Mayer’s haematoxylin from Sigma-Aldrich

Microwave from LG

Microscope cover slips from Menzel-glaser

Microtome blades from Leica

Napthol ASTR-phosphate from Sigma-Aldrich

N,N-dimethyl formamide (DMP) from AppliChem

Olympus BX60 camera with CellSens software from Olympus

Paraformaldehyde (PFA) from Sigma-Aldrich

Pararosaniline from Sigma-Aldrich

Scissor from Gold Coast Hospital

Shandon Citadel 1000 tissue processor from Thermo Fisher Scientific

Sodium citrate dihydrate from Sigma-Aldrich

Sodium nitrate from Merck

Sodium tartrate from Merck

Superfrost plus glass slides from Lomb Scientific

Tissue embedding medium paraffin from Leica

Tweezer from Gold Coast Hospital

Xylene from Merck
Table 2.1 Primers used in realtime-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Length</th>
</tr>
</thead>
</table>
| 18s rRNA| Forward: 5'-CTTAGAGGGACAAGTTGGCG-3’  
Reverse: 5'-ACGCTGAGCCAGTCAGTGTA-3’ | 107 bp |
| E-cad   | Forward: 5'-GAACAGCAGCTACACAGCCCT-3’  
Reverse: 5'-GCAGAAGTGTCCCTTGGGAG-3’ | 76 bp  |
| GAPDH   | Forward: 5'-TGCAACCACCAACTGCTTAGC-3’  
Reverse: 5'-GGCATGGGACTGTGTCATGAG-3’ | 87 bp  |
| MCP-1   | Forward: 5'-TCGCCAGCTATAAGAAGAAATCA-3’  
Reverse: 5'-TGTTCAAGTCTTGGAGTGTG-3’ | 161 bp |
| MMP-2   | Forward: 5'-GACATACATCTTTTGGCTGGAGAC-3’  
Reverse: 5'-TTCAAGGATAAGGCACCTT-3’ | 180 bp |
| MMP-9   | Forward: 5'-CTTCACCTTTCCTGAGAAG G-3’  
Reverse: 5'-CACTTCTTGTCTGTCATAA-3’ | 105 bp |
| MT1-MMP | Forward: 5'-CTCGGGATCTGTCAGGAAATGGAGG-3’  
Reverse: 5'-TTCTCCGTGTCCATCCACTTGGT-3’ | 146 bp |
| N-cad   | Forward: 5'-GACGGTTCCCGCATCCAGAC-3’  
Reverse: 5'-TCGATTGGTGGCACCAGG-3’ | 66 bp  |
| OPG     | Forward: 5'-GGAACCAGAGAGCAGAAATACA-3’  
Reverse: 5'-CCCTGAAAGAATGCTCTACA-3’ | 225 bp |
| RANKL   | Forward: 5'-CAGAAGATGCGACTCAGTCA-3’  
Reverse: 5'-CCCGGATCTGCTCCTGCTCT-3’ | 203 bp |
| Runx2   | Forward: 5'-CCAGATGGGACTGTGGTTACT-3’  
Reverse: 5'-ACGGTTATATGTCAGGGTGAAAC-3’ | 164 bp |
| Snail1  | Forward: 5'-TGCAAGGACTCTTAAATCAAGTACC-3’  
Reverse: 5'-GTGGGATGGCTGCGAC-3’ | 71 bp  |
| TIMP-1  | Forward: 5'-GGAGAGTGCTCTGGCAGTACTTC-3’  
Reverse: 5'-GCTAGTGATGTCAGTGCAAGAGTC-3’ | 100 bp |
| TIMP-2  | Forward: 5'-ACCCCTCTGTGACTTCTGTCG-3’  
Reverse: 5'-GGAGATGTAGCAGGAGTATG-3’ | 129 bp |
| Twist1  | Forward: 5'-TGTCGGCGATCCACTAGC-3’  
Reverse: 5'-TGTCATTTTCTCCTTCTCTGGA-3’ | 63 bp  |
<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Company Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tubulin mouse anti-human monoclonal primary antibody</td>
<td>Abcam</td>
</tr>
<tr>
<td>ALP mouse anti-human monoclonal primary antibody</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>CK mouse anti-human monoclonal primary antibody</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>CD14 rat anti-mouse monoclonal primary antibody</td>
<td>eBioscience</td>
</tr>
<tr>
<td>E-cad mouse anti-human monoclonal primary antibody</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>MCP-1 rabbit anti-human polyclonal primary antibody</td>
<td>Abcam</td>
</tr>
<tr>
<td>MMP-2 mouse anti-human polyclonal primary antibody</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>MMP-9 mouse anti-human polyclonal primary antibody</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>MT1-MMP rabbit anti-human polyclonal primary antibody</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>N-cad rabbit anti-human polyclonal primary antibody</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>OPG rabbit anti-human polyclonal primary antibody</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>RANKL mouse anti-human monoclonal primary antibody</td>
<td>Abcam</td>
</tr>
<tr>
<td>Runx2 rabbit anti-human polyclonal primary antibody</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Snail1 rabbit anti-human polyclonal primary antibody</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>TIMP-1 rabbit anti-human polyclonal primary antibody</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>TIMP-2 rabbit anti-human polyclonal primary antibody</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Twist1 rabbit anti-human polyclonal primary antibody</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>VIM mouse anti-human monoclonal primary antibody</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>PE-conjugated rat anti-mouse secondary antibody</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>HRP-conjugated goat anti-mouse secondary antibody</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>HRP-conjugated goat anti-rabbit secondary antibody</td>
<td>Bio-Rad</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Cell lines and culture conditions

The OSCC cell lines SCC15 and SCC25 were gifts from Associate Professor Nick Saunders (Princess Alexandra Hospital, QLD, Australia). Other OSCC cell lines, HN5 and Tca8113, were kindly supplied by Professor Ming Wei (Griffith University, Australia) and Professor Qian Tao (Sun Yat-sen University, China). Normal dermal epithelial cells (EP) were kindly supplied by Queensland Institute of Medical Research (QIMR, QLD, Australia). The prostate cancer cell line PC3 and the fibrosarcoma cell line HT1080 were supplied by Professor Jin Gao (James Cook University, QLD, Australia). The immortalised human foetal osteoblast cell line of hFOB, and the murine macrophage cell line RAW264.7 were obtained from American Type Tissue Collection (ATCC, Rockville, USA). OSCC cells of SCC15 and SCC25 were maintained in DMEM/F12 or DMEM supplemented with 10% FBS and antibiotics (100 U/mL of penicillin G and 100 mg/mL of streptomycin) at 37°C in an incubator (5% CO₂/20% O₂). Other cells of HN5, Tca8113, RAW264.7, EP and PC-3 were maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/mL of penicillin G and 100 mg/mL of streptomycin) at 37°C in an incubator (5% CO₂/20% O₂). hFOB cells were grown in DMEM/F12 with 10% FBS plus 300 μg/mL genetin (G418) at 34°C in an incubator (5% CO₂/20% O₂), which permits expression of the large T antigen based on the protocol from ATCC.
2.2.2 Primary osteoblast cell culture

Primary osteoblast (OB) cell culture was established using an explant method from normal human cancellous bone of patients, who underwent elective hip/knee replacement surgery in the Gold Coast Hospital (Gold Coast, Australia). Informed consent was obtained from each patient and research protocols were approved by both Human Research Ethics Committee of Griffith University (DOH/01/07/HREC) and Queensland Health. OB cells were cultured in DMEM/F12 with 10% FBS and antibiotics (100 U/mL of penicillin G and 100 mg/mL of streptomycin) at 37°C in an incubator (5% CO₂/20% O₂). The medium was changed every 3 days until cells becoming confluent. OB cells were characterized by immunocytochemical detection of ALP and by Von Kossa staining.

2.2.3 ALP staining by immunocytochemistry

OB cells were fixed with 10% formalin for 10 min and permeabilized by 0.1% Triton X-100 for 5 min. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide for 20 min. Non-specific binding of the antibodies was avoided using 5% BSA in PBS for 30 min. Thereafter, the fixed cells were incubated with the primary antibody of ALP (1:50) overnight at 4°C, and then with the secondary antibodies for 30 min at 37°C. Non-immune serum instead of the primary antibody was used as negative controls. Three washes of PBS were applied before the incubation of primary and secondary antibodies. Sites of binding were visualized using liquid diaminobenzidine (DAB) + substrate + chromogen system (Dako, USA),
counterstained with Mayer's haematoxylin, and photographed by a Nikon OXM1200 digital camera with the Act-1 program.

2.2.4 Von Kossa staining

OB cells at passages 4-6 were plated at a density of 3×10⁵ cells per well in six-well plates and allowed to attach for 24 h. After a consecutive culture for 20 days with medium change every 3 days, the mineral deposition was identified by Von Kossa staining. OB cells were fixed in 10% formalin, rinsed with distilled water, incubated in 1% silver nitrate under UV light for 2 h, developed then stopped by addition of 3% sodium thiosulfate. Fixed calcium deposits in each well were captured using a Nikon OXM1200 digital camera with the Act-1 program.

2.2.5 Differentiation of murine osteoclasts from cells of RAW264.7

Murine osteoclasts were generated from the murine macrophage cells of RAW264.7, which were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/mL of penicillin G and 100 mg/mL of streptomycin) at 37°C in an incubator (5% CO₂/20% O₂). To obtain osteoclasts, these cells were seeded in a 96-well plate at a density of 1.25×10⁴ cells/well, and supplemented with 50 ng/mL of recombinant mouse RANKL on day 1 and day 3. Mature osteoclasts were observed on day 5. Tartrate-resistant acid phosphatase (TRAP) staining was performed to identify these osteoclasts, which were photographed using a Nikon OXM1200 digital camera with the Act-1 program.

2.2.6 Generation of human osteoclasts from CD14⁺ monocytes
Peripheral blood mononuclear cells (PBMCs) were isolated from human blood of healthy volunteers using BD vacutainer cell preparation tubes (CPT) with sodium citrate. All protocols were approved by Griffith University Ethics Committee. After being centrifuged at 1500 g for 30 min, the cell layer on top of the Ficoll-Paque in the CPT was collected, resuspended in 10 ml of α-MEM and centrifuged (1250 rpm, 10 min). CD14⁺ monocytes were purified by incubation with MACS CD14 microbeads for 15 min at 4°C. Cells were washed in CD14⁺ isolation buffer (0.5% FCS; 2 mM EDTA, pH 8) and passed through an MACS magnetic cell separator. Then CD14⁺ monocytes were planted in 24-well plates at 1×10⁵ cells per well in 600 μL of medium (α-MEM, pH 7.4, containing 10% FBS and 1% penicillin/streptomycin), supplemented with the recombinant human cytokines of CSF1 (25 ng/mL) and RANKL (40 ng/mL) for generating osteoclasts. The medium was changed every 3 days and mature osteoclasts appeared in one week. TRAP staining and immunofluorescence of F-actin were used to characterize human osteoclasts.

2.2.7 TRAP staining

TRAP staining of murine or human osteoclasts of cell culture was performed based on the constructions of TRAP staining kit (STrACP, Nanjing Jiancheng). Alternatively, TRAP staining was performed based on the protocol of Associate Professor Nigel Morrsion’s laboratory: cells were fixed with 10% formalin for 10 min, washed once with PBS and treated with ethanol-acetone (50:50 v/v) for 1 min. After another wash with PBS, all cells were incubated with TRAP staining buffer (50 mM sodium L-tartrate disbasic dehydrate; 100 mM sodium acetate; 0.5 mg/mL naphthol AS-MX phosphate; 0.5 mg/mL fast red violet LB salt; pH 5) for 15 min at room temperature.
Those cells positive for TRAP and containing three or more nuclei were considered multinuclear osteoclasts.

2.2.8 F-actin ring staining by immunofluorescence

Human osteoclasts were fixed in 10% formalin, permeabilized with 0.1% Triton X-100 for 10 min and stained in the dark with rhodamine labelled phallodin for F-actin. The stock solution of rhodamine phalloidin was diluted in PBS (1:200). The stained cells were incubated at room temperature for 20 min before the staining solution was removed and washed twice with PBS. Hoechst dye or 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) stain was used for counterstaining with nuclei acid. The stock solution of DAPI was diluted in PBS (1:500). The stained cells were incubated at room temperature for another 5 min, and then washed twice with PBS for analysis. Images with immunofluorescence were taken using a Nikon TP2 digital camera with the NIS program.

2.2.9 Indirect cell co-culture models

Indirect cell co-cultures between OSCC cells and osteoblasts were performed in Chapter 3. SCC15, SCC25, hFOB or OB cells were firstly seeded individually at a density of $1.5 \times 10^6$ cells in 75-cm$^2$ flasks and cultured overnight. The culture medium was then changed to DMEM/F12 without FBS. After a further culture for 48h, supernatant from each cell line was collected and centrifuged at 1500 g, for 20 min at $4^\circ$C to remove cell debris, and used as conditioned medium (CM) in the following
indirect cell co-culture. All cells were plated individually at a density of $2 \times 10^5$ cells per well in 6-well plates. At 80% confluence, cells were washed with PBS, and then all medium was replaced with CM. OSCC cells were treated with CM from osteoblasts and vice versa, controls were treated with CM from the same cell type. After another 48hs of culture, CM, and RNA and protein extracted from the residual cells of each cell line were collected for further detection.

The indirect cell co-culture of CD14$^+$ monocytes and SCC25 cells was performed in Chapter 5 within the 24-well culture plates. Cell groups were arranged as follows: Group 1. CD14$^+$ monocytes only; Group 2. CD14$^+$ monocytes treated with CSF1 (25 ng/mL) and RANKL (40 ng/mL); Group 3. CD14$^+$ monocytes treated with CSF1 (25 ng/mL) and RANKL (40 ng/mL), plus 10% (v/v) conditioned medium (CM) of SCC25 cells; Group 4. CD14$^+$ monocytes treated with CSF1 (25 ng/mL) and RANKL (40 ng/mL), plus 10% (v/v) CM of SCC25-7ND cells. CM (supernatant) was collected as follows: cells were firstly washed with PBS and then all medium was changed into α-MEM without FBS. After 48hs of culture, the entire medium was collected and centrifuged at 1500 g, for 20 min at 4°C to remove any cell debris. After these cells being cultured under the same condition for 7 days, osteoclasts were fixed in 10% formalin and stained for TRAP and F-actin.

2.3.0 TGF-β1 treatment of OSCC cells

To examine effects of TGF-β1 in Chapter 2, equal numbers of OSCC cells of SCC25, HN5 and Tca8113 were plated in serum-free medium, starved for 12h, and treated with TGF-β1 (5 ng/mL) for 0-3 days. Culture medium was changed every day.
2.3.1 Cell proliferation assay

OSCC cells of SCC25, HN5 and Tca8113 were seeded at a density of $5 \times 10^3$ cells/well in 96-well plates, allowed to attach overnight and then treated as above. Absorbance was read at 590 nm on a Biomek plate reader, after addition of 20 μL methylthiazol tetrazolium (MTT, 5 mg/mL) to the wells for 4h incubation, followed by removal of all solution and addition of 150 μL/well of dimethyl sulfoxide (DMSO) to solubilise the cells.

2.3.2 Cell transfection

Cell transfection of SCC25 cells was performed in Chapter 5 using the 7ND vector (Fig.2.1), which was kindly supplied by Associate Professor Kensuke Egashira (Kyushu University, Japan). FugeneHD was used to mix 7ND, and the ratio between them was set at 1:3 (v/v) based on optimization experiments. Briefly, OSCC cells of SCC25 were seeded into a 24-well plate at a density of $5 \times 10^4$ cells/well, with 500 μl of complete medium. After 24h, the cells were ready for transfection and complete medium was replaced by Opti-MEM. Different amount of 7ND (0.2 μg, 0.4 μg, 0.6 μg) was added to Opti-MEM respectively, to which the required amount of FugeneHD was added to a final volume of 20 μL. This 7ND-Fugene mixture was incubated for 15 min at room temperature to allow the transfection complex to form. Then the transfection complex was added dropwise to the cells. After 48h, Opti-MEM was changed back into complete medium. Semi-cloned cells were selected using antibiotic selection with G418.
**Figure 2.1.** The 7ND vector used for cell transfection with SCC25 cells. It was engineered by the insertion of the 7ND sequence into the pcDNA3 vector (Invitrogen, Life Technology), where it was ligated into the BamHI and NotI restriction sites within the polylinker region. It also contains a G418/neomycin resistance cassette for antibiotic selection.

### 2.3.3 Kill curve assay of SCC25 cells by using G418

The G418 kill curve assay was performed to determine at which concentration of G418 100% cell death would be observed for normal SCC25 cells. Briefly, SCC25 cells were seeded in a 24-well plate at a density of 5x10⁴ cells/well, with 400 µL of complete medium. The medium was changed every 3 days by gently rinsing the cells with PBS then adding fresh complete medium, plus fresh administration of G418 at each change. The assay tested a range of concentrations of G418 from 0.2 mg/mL to
1.3 mg/mL, each performed in triplicate for 6 days. The minimum concentration of G418 was chosen when total cell death was observed on day 6 from the range of concentrations of G418.

2.3.4 Establishment of stabilized SCC25 cells with 7ND vector by antibiotic selection

To establish a stable OSCC cell line transfected with 7ND vector, the transiently transfected cells of SCC25 were placed under G418 antibiotic selection. For SCC25 cells transfected with different amount of 7ND (0.2 μg, 0.4 μg, 0.6 μg) after 24 hours, G418 treatment commenced at the minimum concentration of 0.3 μg/mL. After approximately 6 days, the cells were split in half and replated into 24-well plates. Antibiotic administration continued for another three weeks, at which time net growth was observed. Thereafter, the cells from three of the most resistant cultures were removed from the 24-well plate, pooled and then re-seeded by a one in two serial dilution in duplicate over two 24-well plates. After 4 days, these cells with a single colony of cell growth were transferred to a new well in a 12-well plate. The cells were up-scaled further until they were grown in 25 cm² tissue culture flasks. These cells were then lysed for total RNA extraction, cDNA was subsequently synthesized from the RNA and real-time PCR was used to determine the relative gene expression of MCP-1 in all these cells, and the one which had the greatest level (SCC25-7ND) would be used in other experiments.

2.3.5 RNA isolation
RNA isolation was performed using a kit-based method. The PureLing RNA Mini Kit was employed to isolate RNA from small quantities of cells such as those from 24 well plates. RNA was isolated based on kit instructions. RNA was quantified and quality tested by spectrophotometry and gel electrophoresis. All samples were confirmed at 260/280 absorbance ratios between 1.8 and 2.1 and had prominent 18s and 28s bands.

2.3.6 cDNA synthesis

The RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, USA) based on the instructions. Alternatively, the first-strand cDNA was synthesized according to the protocol of Associate Professor Nigel Morrison’s laboratory as shown in the following tables:

Table 2.3 cDNA synthesis reagent protocol

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1 x</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x RT reaction buffer</td>
<td>4 μL</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.4 μL</td>
</tr>
<tr>
<td>OligoDT</td>
<td>1.2 μL</td>
</tr>
<tr>
<td>5 mM dNTPs</td>
<td>2.2 μL</td>
</tr>
<tr>
<td>100 μg/mL random primers</td>
<td>1.2 μL</td>
</tr>
<tr>
<td>Reverse transcriptase (RT)</td>
<td>1 μL</td>
</tr>
<tr>
<td>RNA</td>
<td>8 μL</td>
</tr>
<tr>
<td>Total</td>
<td>20 μL</td>
</tr>
</tbody>
</table>
Table 2.4 Thermocycler protocol for cDNA synthesis

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Repeats</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>70 °C for 30 sec</td>
<td>42 °C for 60 min</td>
<td>4 °C for ∞</td>
</tr>
</tbody>
</table>

2.3.7 Real-time PCR

The synthesised DNA of all cells before and after the treatment was transferred to the real-time PCR reaction mixtures and subjected to the iCycler iQ5 real-time PCR system. Quantitative expressions of genes of Twist1, Runx2, MMP-2, MMP-9, RANKL, OPG, Snail1, E-cad, N-cad, TIMP-1, TIMP-2, MT1-MMP and GAPDH in Chapter 3 and 4 were performed using the iCycler iQ5 real-time PCR system (Bio-Rad, USA) based on the protocol of EXPRESS SYBR GreenER qPCR SuperMixes, Two-Step qRT-PCR Kits. Quantitative expressions of genes of MCP-1 and 18s rRNA in Chapter 5 were performed using the iCycler iQ5 real-time PCR system based on the protocol of Associate Professor Nigel Morrison’s laboratory as shown in the following tables:

Table 2.5 Reagent protocol for real-time PCR

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1 x</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μM primer F+R (MCP-1/18s rRNA)</td>
<td>2 μL</td>
</tr>
<tr>
<td>2x iQ SYBR Green Supermix</td>
<td>10 μL</td>
</tr>
<tr>
<td>Pure H₂O</td>
<td>6 μL</td>
</tr>
<tr>
<td>cDNA</td>
<td>2 μL</td>
</tr>
<tr>
<td>Total</td>
<td>20 μL</td>
</tr>
</tbody>
</table>
Table 2.6 Thermocycler protocol for real-time PCR

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Repeats</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95 °C for 2.5 min</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>95 °C for 10 sec</td>
<td>58 °C for 10 sec</td>
<td>72 °C for 25 sec</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>60 °C for 5 sec</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The data were normalized to the internal control of GAPDH or 18s rRNA to obtain ΔCt. The final amount of genes of interest relative to untreated samples was reported by the $2^{-\Delta\Delta C_t}$ method. Primers used in this study are listed in Table 2.1.

2.3.8 Gelatine zymography

Zymogenic activities of both MMP-2 and MMP-9 were analysed by gelatine zymography. CM from all cells before and after the treatment was collected as above. CM of HT1080 cells served as the positive control. Equivalent amounts of protein per sample were mixed with non-reducing sample buffer (62.5 mM Tris-HCl, pH 6.8; 4% SDS; 25% glycerol; 0.01% Bromophenol Blue) and electrophoresed on 10% precast denaturing SDS polyacrylamide gel with gelatine (Bio-Rad Lab, CA, USA). Gels were washed in the commercial renaturation solution (2.5% Triton X-100) for 30 min at room temperature and incubated in development solution (50 mM Tris; 200 mM NaCl; 5 mM CaCl$_2$; 0.02% Brij-35) at 37°C for 40 h. Finally, gels were stained with Coomassie Brilliant Blue R-250 for 1 h at room temperature and progressively destained until clear bands appeared against the blue background. Representative photos were either scanned using a scanner, or taken using a Panasonic digital camera.
2.3.9 Western blotting

Cell lysates before and after the treatment were extracted using RIPA lysis buffer (10mM Tris-HCl; 1 mM EDTA; 1% sodium dodecyl sulphate; 1% Nonidet P-40; 1:100 proteinase inhibitor cocktail; 50 mM β-glycerophosphate; 50 mM sodium fluoride). The protein concentration was determined with a BCA Protein Assay Kit. Briefly, 40 µg of protein was subjected to SDS–PAGE with 10% polyacrylamide gels. The gels were transferred to PVDF membranes, and then blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 2 h at room temperature. The membranes were incubated with each primary antibody, Twist1 (1:200), Runx2 (1:200), Snail1 (1:200), MMP-2 (1:500), MMP-9 (1:500), E-cad (1:200), N-cad (1:100), TIMP-1 (1:200), TIMP-2 (1:200), MT1-MMP (1:200), RANKL (1:500), OPG (1:200) and α-tubulin (1:3000) overnight at 4°C. Membranes were then washed twice and incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies (1:3000) for 1 h at room temperature. Subsequently, the protein bands were detected by SuperSignal WestPico chemiluminescent substrate (Thermal Scientific) and visualised using VersaDoc-MP Imaging Systems.

2.4.0 Haematoxylin & eosin staining

Archival blocks from 12 patients whose OSCC showed invasion of bone were kindly supplied by Professors Glenn Francis and Jane Dahlstrom from Royal Brisbane and Women Hospital (Brisbane, QLD, Australia) and The Canberra Hospital (Canberra, ACT, Australia), following the approval of Ethics Committee of Queensland Health and Australian Capital Territory (ACT) Human Research Ethics Committee. Serial
paraffin tissue sections (5 µm thickness) were dewaxed in Xylene (2×10 min), and rehydrated with 100%, 90%, 70% ethanol respectively (5 min). After being rinsed with distilled water, sections were stained by Harris haematoxylin (5 min), differentiated with 1% HCl in 70% ethanol for 1-2 dips, and washed in running tap water for 15 min. Afterwards, sections were stained with alcoholic eosin solution for 1 min, dehydrated with 70%, 90%, 100% ethanol respectively (5 min), and cleared with Xylene (2×10 min). All sections were finally mounted with mounting medium and microscope cover slips.

2.4.1 Immunohistochemistry

These archival blocks from 12 patients whose OSCC showed invasion of bone described above were examined to validate expressions of targeted molecules. Serial paraffin tissue sections (5 µm thickness) were dewaxed, rehydrated and treated with 0.3% hydrogen peroxide in PBS. Antigen retrieval was performed by heating sections in a microwave oven (2×4 min) with 0.2% citrate buffer (pH 6). After non-specific binding was blocked with 5% BSA in PBS for 30 min, sections were incubated with primary antibodies of MMP-2 (1:50), MMP-9 (1:50), Twist1 (1:80), RANKL (1:100), OPG (1:80), CK (1:50), VIM (1:50), E-cad (1:50), Snail1 (1:80) and MCP-1 (1:50) overnight at 4°C. Sections were then treated with the anti-mouse/rabbit secondary antibodies (Envision + Systems) (Dako, USA) for 30 min, followed by diaminobenzidine (DAB) detection solution for a few minutes at room temperature. Primary antibodies were replaced by non-immune serum as negative controls. Sections were counterstained with Mayer’s haematoxylin, dehydrated, and mounted with mounting medium. The final results were visualized by light microscopy and
photographed using an Olympus Bx60 digital camera with the CellSens software. Staining was interpreted without prior knowledge of clinical-pathologic parameters by 2 observers.

2.4.2 *In vivo* animal model of bone invasion by OSCC

Balb-c nude mice were purchased from the Animal Resources Centre (ARC, Australia). These mice were housed in the animal facility of Griffith University Gold Coast Campus, and cared by the animal house staff. All protocols were reviewed and approved by Griffith University Animal Ethics Committee (MSC/06/11/AEC). At 6-7 weeks old, these mice were used in Chapter 5 to develop an animal model of bone invasion by OSCC *in vivo*. Under sterile conditions, OSCC cells of SCC25 (6×10⁶/100 μL) were injected subcutaneously overlaying the calvaria. Mice were randomly divided into three groups (n=6/group): the negative control group received PBS (Group 1); the positive control group received cells of SCC25 (Group 2); the experimental group received cells of SCC25-7ND (Group 3). All animals were sacrificed after 6 weeks of tumour development. Tumours and calvaria were removed and fixed in 4% PFA for histological and immunohistochemical analysis.

2.4.3 Flow cytometry analysis of bone marrow cells of animal experimentation

Bone marrow cells (BMCs) from mice in different groups were taken out from tibia on Week 6. The CD14 subpopulation of BMCs from mice in different groups were evaluated by incubating 1×10⁶ cells with the anti-CD14 antibody in PBS at 4°C for 30 min. Thereafter, cells were stained with PE-conjugated rat anti-mouse secondary
antibody. Flow cytometry analysis (FACS) was carried out on a FACScan flow cytometer. The unstained cells were gated out and data acquisition with analysis was done using CellQuest software.

2.4.4 Tissue samples preparation for animal experimentation

PFA-fixed tumour specimens collected from these nude mice in different groups were embedded in paraffin by using Shandon Citadel 1000 tissue processor. Serial 5 μm paraffin sections were cut on Leica RM 2235 rotary microtome and stained with hematoxylin and eosin (H&E). Immunohistochemical staining of the sections was performed by incubation of serial sections with the primary antibody of MCP-1 (1:80) overnight followed by HRP labelled secondary antibody and DAB staining. Specimens treated with non-immune serum served as negative controls.

To perform histochemical staining for the tumour-bearing calvaria, all calvarias were firstly decalcified in 10% EDTA (pH 7.4) for 2 weeks and then processed for paraffin embedding. Serial 5 μm sections of paraffin embedded calvaria were stained by both H&E and TRAP. TRAP staining of murine osteoclasts of tissue sections was performed based on the protocol of Associate Professor Nigel Morris's laboratory. Briefly, Serial tissue sections were dewaxed in Xylene (2×10 min), and rehydrated with 100%, 90%, 70% ethanol respectively (5 min). After being rinsed with distilled water, sections were incubated with TRAP staining solution (40 mg/mL Pararosaniline-HCl; 40 mg/mL sodium nitrate; 10 mg/mL Napthol ASTR-phosphate-DMF; 10 mg/mL sodium tartrate; pH 5) for 30 min, washed with distilled water (3×3min) and counterstained with Mayer’s haematoxylin for 1 min then washed with
5 min in running tap water. Sections were dried overnight and then mounted with
coverslip using permanent mounting medium. Histomorphometric analysis of TRAP-
positive osteoclast numbers at the tumor-bone interface was performed.

2.4.5 Statistical analysis

Results were presented as mean ± standard error (M±SE) of at least 3 independent
experiments. Data analysis was performed using SAS (SAS 8.1, USA) and SPSS
software (SPSS 20.0, IBM, USA). A paired Student t test was used to compare two
means. One way analysis of variance (ANOVA) was applied to compare two or more
means, followed by Student-Newan-Keuls (S-N-K) test. A p value of less than 0.05
was regarded as statistically significant.
Chapter 3

Indirect co-culture between osteoblasts and OSCC cells
3.1 Introduction

Oral cancer is the sixth most common cancer worldwide, with a poor five-year survival rate of only 50 to 60%: more than 90% of oral malignancies are oral squamous cell carcinomata (OSCC). Lesions arising in the gingiva, retromolar trigone, buccal mucosa or floor of mouth may extend to bone of the maxilla and/or the mandible. An understanding of the mechanisms of bone invasion is essential in minimising spread and in planning surgical ablation, especially for guiding maxilla or mandible preservation surgery.

Although little is known as to whether OSCC cells have the ability to resorb bone themselves, it is regularly observed that osteoclasts are much involved in this event. Generation of osteoclasts in the presence of tumour cells is likely to be due to an indirect effect involving stimulation of osteoblasts. Osteoblasts produce a family of tumour necrosis factor (TNF)-like proteins and receptor (TNFR) molecules: these include osteoprotegerin (OPG), receptor activator of nuclear factor (NF)-κB (RANK) and RANK ligand (RANKL), any or all of which may interact in the regulation of osteoclast function. While RANKL is an inducer of osteoclasts, formed through its interaction with RANK, OPG is a soluble decoy receptor and acts as an inhibitor. Therefore, the relative expressions of RANKL and OPG are crucial to determining the turnover from bone formation to bone destruction.

Recently, several signal pathways initiated by products of neoplastic keratinocytes themselves are reported to affect osteoclast function: the latter include proteases, cytokines, and growth factors. Matrix metalloproteinases (MMPs) are the main
proteases responsible for digestion of fibrillar and non-fibrillar collagens, elastin, gelatine and proteoglycans. These proteolytic enzymes not only facilitate the entry of malignant keratinocytes into the soft tissues or marrow spaces within bone, but also act on bone cells to effect their differentiation and maturation.

Studies of the mechanisms of distant metastases to bone, which occur particularly with neoplasms of the breast, prostate, thyroid, kidney and lung, encourage us to rethink the mechanisms of local bone invasion by other carcinomas, including those arising in the mouth. The phenomenon of osteomimicry has been described frequently, whereby tumour cells acquire osteomimetic or osteoblast-like characteristics and express molecules known to be involved in bone homeostasis. Recently, Yuen et al found that the so-called “medium of osteogenic induction” could up-regulate expressions of Twist1 and Runx2 in prostate cancer cells after 12 days incubation, which might facilitate survival of these cells within bone. Twist1 is a negative transcription factor which inhibits osteoblast differentiation, maintaining these cells in the osteoprogenitor state. Twist1 would competitively interact with Runx2, which acts as a master regulator, essential in the initial differentiation of mesenchymal cells into osteoblasts. In addition, these two key molecules of bone homeostasis have been reported to be activators of several members of the MMP family. For example, the over expression of Twist1 has been shown to increase the invasion of gastric carcinoma cells, by promoting MMP-2 secretion, and high levels of Runx2 have been shown to increase expressions of MMP-9 and MMP-13 in prostate cancer cells. Such activities increase the propensity of these particular neoplastic cells to metastasise to bones. However these mechanisms have not been studied in respect of OSCC.
In order to investigate the mechanisms of bone invasion by OSCC, the present study aims to investigate the crosstalk between cancer cells and bone cells. We hypothesized that MMPs, especially MMP-2 and/or MMP-9, interacting with Twist1, Runx2, RANKL and OPG, play important roles in the invasion of bone by OSCC.

3.2 Materials

DMEM/F12 medium, foetal bovine serum (FBS), trypsin-EDTA and the primary antibody of anti-alkaline phosphatase (ALP) were purchased from Life Technologies (USA). Other primary antibodies, anti-Twist1, anti-Runx2 and anti-OPG were purchased from Santa Cruz Biotechnology (USA); anti-MMP-2 and anti-MMP-9 were purchased from Cell Signalling Technology (USA); and anti-RANKL and anti-α-tubulin from Abcam (USA). The secondary antibodies goat anti-mouse IgG and goat anti-rabbit IgG were supplied by Bio-Rad Laboratories (USA).

3.3 Methods

3.3.1 Cell lines and culture conditions

The OSCC cell lines SCC15 and SCC25 were kind gifts of Associate Professor Nick Saunders (Princess Alexandra Hospital, Brisbane). The immortalised human foetal osteoblast cell line hFOB was obtained from American Type Tissue Collection (ATCC, Rockville, MD). OSCC cells were maintained in DMEM/F12 with 10% FBS and antibiotics (100 U/mL of penicillin G and 100 mg/mL of streptomycin) at 37°C in
an incubator (5% CO₂/20% O₂). hFOB cells were grown in DMEM/F12 with 10% FBS plus 300 μg/mL geneticin at 34°C.

### 3.3.2 Primary osteoblast cell culture

Primary osteoblast (OB) cell culture was established using an explant method from normal human cancellous bone of patients, who underwent elective hip/knee replacement surgery in the Gold Coast Hospital (Gold Coast, Australia). Informed consent was obtained from each patient and research protocols were approved by Human Research Ethics Committee of Griffith University and Queensland Health. OB cells were cultured in DMEM/F12 supplemented with 10% FBS and antibiotics at 37°C in an incubator. The medium was changed every 3 days. OB cells were characterized by immunocytochemical detection of ALP and by Von Kossa staining.

### 3.3.3 Indirect co-culture

SCC15, SCC25, hFOB or OB cells were firstly seeded individually at a density of 1.5×10⁶ cells in 75-cm² flasks and cultured overnight. The culture medium was then changed to DMEM/F12 without FBS. After a further 48hs’ culture, supernatant from each cell line was collected and centrifuged at 1,500 g, for 20 min at 4°C to remove cell debris, and used as conditioned medium (CM) in the following indirect cell co-culture. All cells were plated individually at a density of 2×10⁵ cells per well in 6-well plates. At 80% confluence, cells were washed with phosphate buffered saline (PBS) and the medium was replaced with CM. Cancer cells were treated with CM from osteoblasts and vice versa, controls were treated with CM from the same cell type.
After another 48hs’ culture, CM, and mRNA and protein extracted from the residual cells of each cell line were collected for the experiments described below.

### 3.3.4 Gelatine zymography

CM from all cells before and after the indirect co-cultures were analysed by gelatine zymography. CM of HT1080 cells served as the positive control. Equivalent amounts of protein per sample were mixed with non-reducing sample buffer (62.5 mM Tris-HCL, pH 6.8; 4% SDS; 25% glycerol; 0.01% Bromophenol Blue) and electrophoresed on 10% precast denaturing SDS polyacrylamide gel with gelatine (Bio-Rad Lab, CA, USA). Gels were washed in the commercial renature solution (2.5% Triton X-100) for 30 min at room temperature and incubated in development solution (50 mM Tris; 200 mM NaCl; 5 mM CaCl2, 0.02% Brij-35) at 37°C for 40 h. Finally, gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, USA) for 1 h at room temperature and progressively destained until clear bands appeared against the blue background.

### 3.3.5 Western blotting

Following the indirect co-culture, cell lysates were extracted using RIPA lysis buffer (10 mM Tris-HCL; 1 mM EDTA; 1% sodium dodecyl sulphate; 1% Nonidet P-40; 1:100 proteinase inhibitor cocktail; 50 mM β-glycerophosphate; 50 mM sodium fluoride). The protein concentration was determined with a BCA Protein Assay Kit (Thermo Scientific, USA). Briefly, 40 μg of protein was subjected to 10% SDS-PAGE precasting gel (Bio-Rad Lab, CA, USA). The gels were transferred to PVDF
membranes, and then blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 2 h at room temperature. The membranes were incubated with each primary antibody, MMP-2 (1:500), MMP-9 (1:500), Twist1 (1:200), Runx2 (1:200), RANKL (1:500), OPG (1:200) and α-tubulin (1:3000) overnight at 4°C. Membranes were then washed twice and incubated with horseradish peroxidase-conjugated secondary antibodies (1:3000) for 1 h at room temperature. Subsequently, the protein bands were detected by enhanced chemiluminescence (ECL) and visualised using VersaDoc-MP Imaging Systems (Bio-Rad).

3.3.6 Real-time PCR

After the indirect co-culture, total RNA from OB or OSCC cells was extracted using the PureLing RNA Mini Kit (Life Technologies, USA). The RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, USA). Quantitative expressions of RANKL, OPG, MMP-2, MMP-9, Twist1, Runx2 and GAPDH genes were performed using EXPRESS SYBR GreenER qPCR SuperMixes, Two-Step qRT-PCR Kits (Life Technologies, USA) and the iCycler iQ5 real-time PCR system (Bio-Rad, USA). The data were normalized to the internal control, GAPDH, to obtain ΔCt. The final amount of gene of interest relative to untreated samples was reported by the 2^−ΔΔCt method. Primers used in this study are listed in Table 2.1.

3.3.7 Immunohistochemistry
To validate expressions of these targeted molecules, we examined archival blocks from 12 patients whose OSCC showed invasion of bone. Serial tissue sections (5 μm thickness) were dewaxed, rehydrated and treated with 0.3% hydrogen peroxide in PBS. Antigen retrieval was performed by heating sections in a microwave oven (2×4 min) in 0.2% citrate buffer (pH=6.0). After non-specific binding was blocked with 5% BSA in PBS for 30 min, sections were incubated with primary antibodies of MMP-2 (1:50), MMP-9 (1:50), Twist1 (1:80), RANKL (1:100) and OPG (1:80) overnight at 4°C. Sections were then treated with the anti-mouse/rabbit secondary antibodies (Envision™ + Systems) for 30 min, followed by diaminobenzidine (DAB) detection solution (Dako, Botany, Australia) for a few minutes at room temperature. Primary antibodies were replaced by non-immune serum as negative controls. Sections were counterstained with Mayer’s haematoxylin, dehydrated, and mounted with DPX (BDH Laboratory, Poole, England). The final results were visualized by light microscopy (Leitz Laborlux S, Germany) and photographed using a Nikon OXM1200 digital camera with the Act-1 program.

3.3.8 Statistical analysis

Data analysis was performed using the SAS program (SAS version 8.1, USA). A paired Student t test was used to compare two means. A p value of less than 0.05 was regarded as significant.

3.4 Results
3.4.1 Zymogenic activity changes after indirect co-culture of OSCC and hFOB cells

Gelatine zymography showed that the activities of MMP-2 and MMP-9 were increased in OSCC cells treated with CM of hFOB. MMP-9 was increased while MMP-2 was decreased in hFOB treated with CM of OSCC cells (Fig. 3.1). Preliminary studies using primary human dermal fibroblast cells as a negative control revealed no differences (data not shown).

![Gelatine zymography results](image)

**Figure 3.1.** Gelatine zymography results of the indirect co-culture of OSCC (SCC15 and SCC25) and hFOB cultured cells. The zymogenic activities of MMP-2 and MMP-9 were increased in OSCC cells treated with CM of hFOB (Lanes 2 and 4); MMP-9 was increased while MMP-2 was decreased in hFOB treated with CM of OSCC cells (Lanes 6 and 7). HT1080 cells were used as a positive control. This figure shown is typical of three independent experiments.
3.4.2 Protein level changes after indirect co-culture of OSCC and hFOB cells

Using western blotting, expression of MMP-2 at the protein level was increased, while MMP-9 was decreased, in OSCC cells after indirect co-culture. OSCC cells showed increased expression of Twist1, but no change in the expression of Runx2. For hFOB cells, RANKL protein level was increased, while OPG protein level was decreased. MMP-9 protein level was increased while no change was detected for MMP-2 protein level (Fig. 3.2).

![Western blotting analysis](image)

**Figure 3.2.** Western blotting analysis of the indirect co-culture of OSCC and hFOB cells. MMP-2 protein level was increased, while MMP-9 was decreased in both OSCC cell lines. Twist1 protein expression was increased while no change to protein expression of Runx2 was found. In hFOB cells, RANKL protein expression was increased while OPG was decreased when cultured with CM of the OSCC cell lines: MMP-9 expression was increased while MMP-2 had no change. These results represent three independent experiments.
3.4.3 Primary osteoblast cells showed osteogenic properties

Primary osteoblast (OB) cells displayed features of typical fibroblast-like cells. Immunocytochemistry demonstrated that ALP staining was mostly localised in the cytoplasm (Fig.3.3A-B). After 20 days continuous culture in vitro, Von Kossa staining revealed deposition of calcium salts, reflecting their osteogenic properties (Fig.3.3C-D).

Figure 3.3. Identification of the primary OB cells. (A) ALP staining was mostly localised in cytoplasm (arrow, DAB, bar = 25μm). (B) No staining was found in the negative control, non-immune serum was used to replace the primary antibody (bar = 25μm). (C) Various dark calcium deposits appeared in the culture plate after 20 days’ continuous culture (arrow, bar = 100μm). (D) No calcium deposits were found in the negative control, PBS was used instead of 1% silver nitrate (bar = 100μm).
3.4.4 mRNA level changes after indirect co-culture of OSCC and OB cells

Real-time PCR showed that the RANKL/OPG ratio was significantly increased in OB cells treated with CM of SCC25 ($p<0.05$), while CM of SCC15 had no obvious effect ($p>0.05$). Meanwhile, the expression of MMP-2 was decreased without significance ($p>0.05$), and MMP-9 was significantly increased ($p<0.05$). For OSCC cells, the expression of Twist1 was significantly increased after being treated with CM of OB cells ($p<0.05$), while Runx2 was not changed ($p>0.05$). MMP-2 was significantly increased ($p<0.05$), and MMP-9 was decreased without significance ($p>0.05$) (Fig.3.4).

3.4.5 Immunohistochemical analysis of target molecules in human OSCC samples

H&E staining of sections of OSCC from 12 patients with bone invasion showed an infiltrative pattern\(^3\): cancer cells invaded the bone, and osteoclasts had accumulated in resorption lacunae (Fig.3.5A). IHC showed that MMP-2 was weakly expressed in the cytoplasm of OSCC cells, while MMP-9 was strongly expressed within the cytoplasm of OSCC cells and also weakly in the cytoplasm of osteoclasts (Fig.3.5B-C). For Twist1, weak cytoplasmic expression was found in OSCC cells, and stronger cytoplasmic expression in osteoclasts (Fig.3.5D). The expression of RANKL was similar to MMP-9, while weak expression of OPG could be detected in cytoplasm of OSCC cells with no staining in the osteoclasts (Fig.3.5E-F). Control sections were negative (data not shown).
Figure 3.4. Real-time PCR results of the indirect co-culture of OSCC and OB cells. The RANKL/OPG ratio was significantly increased in OB treated with CM of SCC25 $(p<0.05)$, while CM of SCC15 had no similar effect $(p>0.05)$. The expression of MMP-2 was decreased without significance $(p>0.05)$, and MMP-9 was significantly increased $(p<0.05)$. For OSCC cells, the expression of Twist1 was significantly increased after being treated with CM of OB $(p<0.05)$, while Runx2 was not changed $(p>0.05)$. MMP-2 was significantly increased $(p<0.05)$, and MMP-9 was decreased without significance $(p>0.05)$ in both of SCC15 and SCC25. Data are shown as mean ± standard deviation of three independent experiments $(^*, p<0.05)$. 
Figure 3.5. Immunohistochemical analysis of targeted molecules in sections from OSCC with bone invasion. (A) H&E staining demonstrated an infiltrative pattern of bone invasion by OSCC. (B) MMP-2 was weakly expressed in the cytoplasm of OSCC cells (DAB, bar = 25μm). (C) MMP-9 was strongly expressed in the cytoplasm of OSCC cells and weakly in the cytoplasm of osteoclasts (DAB, bar = 25μm). (D) Weak Twist1 expression was found in cytoplasm of OSCC cells, with stronger expression in the cytoplasm of osteoclasts (DAB, bar = 25μm). (E) The expression of RANKL was similar to that of MMP-9 (DAB, bar = 25μm). (F) Weak expression of OPG was detected in the cytoplasm of OSCC cells and no expression was seen in osteoclasts (DAB, bar = 25μm).
3.5 Discussion

The co-culture model is frequently used in studies seeking to understand the mechanisms of cancer progression and in trials of anti-cancer drugs *in vitro*. Using this model, Okamoto et al showed that CM from cells of the BHY cell line - derived from an OSCC and shown to have bone invasion properties - markedly promoted pit formation in cultures of osteoclasts growing on bone chips; furthermore, these effects could be inhibited by anti-interleukin (IL)-6 antibody.\textsuperscript{53} Ishikuro et al found, also in co-culture experiments, that, BHY cells stimulated expression of RANKL mRNA in mouse osteoblasts.\textsuperscript{47} In comparison to these cytokine studies, less has been reported about the roles of proteases, especially the family of MMPs, in bone invasion by OSCC. The gelatinases MMP-2 and MMP-9, are not only involved in the degradation of bone matrix, but also participate in the recruitment and coordination of osteocytes, osteoblasts and osteoclasts.\textsuperscript{33} Previous work from our group has shown that, of 41 cases of clinically lymph node-positive tongue cancer, 71% and 79% of primary tumours expressed MMP-2 and MMP-9, respectively.\textsuperscript{120} For the other 20 cases of lymph node-negative tongue cancer, 45% and 40% of primary tumours also expressed MMP-2 and MMP-9. In order to explore their roles in the progression of OSCC, we further found that increased expressions of MMP-2 and MMP-9 in OSCC cells, were associated with epithelial-mesenchymal transition (EMT) triggered by transforming growth factor-β1 (TGF-β1) *in vitro*.\textsuperscript{76}

In the present study, using indirect co-culture, we asked whether the invasive phenotype of OSCC cells could be promoted, specifically by studying the expression of MMP-2 and MMP-9. After treatment with CM of hFOB cells, the zymogenic
activities of MMP-2 and MMP-9 were increased in OSCC cells. However, western blotting showed that MMP-2 protein level was increased while MMP-9 was decreased in both of SCC15 and SCC25 cells. Regulation of MMP-2 and MMP-9 is a complex process, operating at the levels of gene expression, post-translational stabilization and zymogen activation. Furthermore, MMPs are also controlled by tissue inhibitors of metalloproteinases (TIMP), which bind to the carboxy-terminus to inhibit their activities; for example, TIMP-2 binds MMP-2 and TIMP-1 binds MMP-9. Therefore, the apparent inconsistencies in MMP-2/9 zymogen activities and protein quantities observed in the present experiments may represent different mechanisms of activity control.

To investigate whether Twist1 and Runx2 were involved, we investigated both gene activity and changes in the protein levels of these markers in our OSCC cell lines. Expression of Twist1 was increased in both SCC15 and SCC25 cell lines. Twist1 has recently been found to promote invasion of human hepatocellular carcinoma (HCC) via increased activities of MMPs. Furthermore, the BHY cell line, which has bone invasive properties, has been shown to have high levels of Twist1 expression. It has been reported that Twist1 would suppress Runx2 to inhibit osteoblast differentiation at an early stage of bone formation. In our study we found that increased expression of Twist1 in OSCC cells had no effect on the expression of Runx2. This was surprising since Runx2 has been shown to act as a transcription factor able to mediate its control genes, especially MMP-9. The reasons for the lack of response of Runx2 in our study are unclear, other transcriptional pathways may have been activated to block the function of Runx2. Investigating these underlying pathways is the focus of future work.
Regulation of osteoclastogenesis involves a pathway containing three essential molecules, RANK, RANKL and OPG. The ratios between the levels of expression of RANKL and OPG are critical in the regulation of osteoclast function. When RANKL expression is enhanced relative to OPG, it is able to bind RANK on osteoclast precursors and induce them to differentiate into mature osteoclasts. Our study showed that after treatment with CM of OSCC cells, the protein level of RANKL was increased while OPG was decreased in hFOB cells, leading to an increased RANKL/OPG ratio; this may tip the balance in favour of osteoclast activation and bone resorption. Meanwhile, the expression of MMP-9 was decreased while MMP-2 was unchanged. As MMP-9 is enriched in osteoclast precursors and is involved in the cleavage of chemokines to molecules which recruit further osteoclasts, the changes we have observed in these molecules suggest differentiation of osteoclasts and thus bone resorption. This could be a component of the uncoupling of bone formation and bone resorption which has been shown to occur in cancer progression.

In addition to the use of an established osteoblast cell line, we also generated primary osteoblast cultures, and confirmed their osteogenic nature with two conventional techniques. We then utilized the indirect co-culture model to investigate if culture supernatant from OSCC cells upregulated mRNA levels of the genes involved in our earlier studies. Although the expression patterns were similar to those observed in the indirect co-culture of OSCC and hFOB cells, some important differences were observed. For example, OB cells, treated with CM from SCC25 cultures showed an increased RANKL/OPG ratio; however, when treated with CM of SCC15 cultures, there was no such effect. Furthermore, the expression of MMP-9 was increased, while
MMP-2 decreased in OB cells treated with CM of OSCC cells. In OSCC cells, treated with CM of OB cells, the expression of Twist1 was increased, but the CM had no effect on Runx2. MMP-2 was increased, but MMP-9 was decreased in co-cultures of both of SCC15 and SCC25. The reasons for these differences are currently unclear. SCC15 and SCC25 are OSCC cell lines with distinct properties; for example, SCC25 has been shown to proliferate faster than SCC15. The expression of focal adhesion kinase (FAK) is also found in SCC25, which may indicate a more invasive potential. However, whether these characteristics of these particular cells affect expressions of the molecules we targeted here is unknown.

To seek further confirmation of our hypothesis, we examined paraffin embedded tissue of 12 cases of OSCC with bone invasion, and sought information on the expression of our targeted molecules by immunohistochemistry. H&E staining showed the infiltrative pattern of bone invasion with osteoclasts located in resorption lacunae. MMP-2 was weakly expressed in tumour cells, while MMP-9 was clearly localized within the cytoplasm of the malignant keratinocytes. For Twist1, weak staining was found in tumour cells, while stronger cytoplasmic expression was seen in osteoclasts. The staining of RANKL was similar to MMP-9, while weak staining of OPG was found in OSCC cells and no staining was seen in osteoclasts. Therefore, this higher RANKL/OPG ratio indicated the balance between bone formation and bone resorption was altered and in favour of bone resorption by OSCC. Compared with the above cell line studies in vitro, these results also indicate a more complex regulation mechanism of these molecules in vivo.
In summary, we have found that CM of osteoblasts promotes invasive properties of OSCC cells via modulating expressions of MMP-2 and MMP-9; CM of OSCC cells increases the RANKL/OPG ratio in osteoblasts to induce the formation of osteoclasts, consistent with increasing their resorbtive properties. Clinical samples showed that nearly all these markers were indeed present in both the malignant keratinocytes and in osteoclasts. It appears that cross-talk between these different cell types is a real phenomenon in cancer biology, enhancing the process of bone invasion. Understanding, and ultimately interfering with these pathways, may provide therapeutic approaches to prevent bone invasion by these cancers.
Chapter 4

Association of epithelial-mesenchymal transition with bone invasion by OSCC
4.1 Introduction

Bone invasion is one of the most frequent complications of OSCC, especially those arising in the retromolar trigone, buccal sulci, gingiva, floor of mouth and hard palate. The incidence is as high as 56% of investigated patients.\textsuperscript{131-133} Bone involvement contributes to increased morbidity, higher recurrence and mortality rates.\textsuperscript{19,134} Despite improvements in current treatment modalities - surgery, radiotherapy and adjunctive chemotherapy - cure rates for these patients remain low. Fortunately, however, there have been considerable advances in understanding the molecular mechanisms of the process of neoplasia, so that the possibility of individualised biotherapies is increasingly recognised. Understanding and ultimately interfering with the molecules involved in bone invasion may enhance such therapeutic approaches.\textsuperscript{135,136}

When epithelial neoplasms metastasise to bone, a vicious circle is established between the malignant cells themselves, and bone tissue. This also occurs when carcinomata invade bone directly, such as with oral cancer.\textsuperscript{137,138} On the one hand, tumour cells secrete multiple factors to alter the bone environment and induce the formation of osteoclasts; while on the other hand, osteoclasts release growth factors from the bone matrix, which then stimulate tumour growth and further accelerate bone destruction.\textsuperscript{95,139} Of these, transforming growth factor-\(\beta\) (TGF-\(\beta\)), which has been widely studied, is a major bone-derived factor responsible for driving this vicious circle. TGF-\(\beta\), along with activins, inhibins, and bone morphogenetic proteins (BMP), are members of the TGF-\(\beta\) super family.\textsuperscript{140} In humans, three isoforms of TGF-\(\beta\) have been identified: TGF-\(\beta\)1, TGF-\(\beta\)2 and TGF-\(\beta\)3.\textsuperscript{141} Normally, TGF-\(\beta\) controls tissue homeostasis by limiting cell proliferation. For cancer cells, it can become an
oncogenic factor to induce proliferation, invasion and immunosuppression during tumour progression.\textsuperscript{142,143} Moreover, TGF-β is implicated in bone metastasis from several solid tumours.\textsuperscript{144} For example, Mohammad et al showed that the inhibitor of TGF-β receptor I (TβRI) kinase, effectively reduced osteolytic lesions and tumour burden in mice with malignant melanoma.\textsuperscript{145} Ganapathy et al demonstrated that two types of TGF-β pathway antagonists (1D11 and LY2109761) significantly decreased metastasis to lung and bone of nude mice, injected with cells of human basal type breast cancer.\textsuperscript{146} As for bone invasion by OSCC, a recent study found that TβRI was expressed by 18 of 21 patients with gingival SCC, and the inhibitor of TβRI greatly reduced the bone destruction caused by OSCC cells \textit{in vitro}.\textsuperscript{22} Additionally, Prime et al observed that the OSCC cell lines BICR31, BICR56, which were resistant to inhibitory effects of TGF-β1, formed significantly more primary tumours, with high incidence of mandibular invasion, when injected through the floor of the mouth of athymic mice.\textsuperscript{66} Taken together, these studies suggest that TGF-β may be a worthy target for therapeutic intervention in cases of oral cancer in man.

In our previous study,\textsuperscript{147} we used an indirect cell co-culture model to explore the cross-talk between osteoblasts and OSCC cells. We found that conditioned medium (CM) of osteoblasts induced osteomimicry in the OSCC cells, for the osteoblast transcription factor, Twist1, was up-regulated in these cells after such treatment (Fig.4.1A). The expression of MMP-2 protein level was increased, while MMP-9 was decreased. Furthermore, immunohistochemical staining of Twist1, MMP-2 and MMP-9 was observed in clinical samples of OSCC patients with bone invasion (Fig.4.1B). We speculate that these changes in gene expression may be caused by growth factors in the CM, which needs further investigation. Since TGF-β is present in significant
amounts in the bone microenvironment, and has been proven to induce EMT in various epithelial cells *in vitro*, we designed the present study to determine the effects of TGF-β1 on OSCC cells and the way they interact with osteoclasts.

### 4.2 Materials

Recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, USA). DMEM medium, foetal bovine serum (FBS), trypsin-EDTA, anti-CK, and anti-E-cad were purchased from Life Technologies (USA). Anti-Twist1, anti-Snaill, anti-MT1-MMP, anti-tissue inhibitors of MMP (TIMP1), anti-TIMP2 and anti-OPG were bought from Santa Cruz Biotechnology; while anti-RANKL and anti-α-Tubulin were from Abcam (USA). The remaining primary antibodies, anti-VIM and anti-N-cad were purchased from Life Technologies (USA) and Cell Signalling (USA) respectively. Secondary antibodies, goat anti-mouse IgG and goat anti-rabbit IgG were from Bio-Rad Laboratories (USA).

### 4.3 Methods

#### 4.3.1 Cell culture

OSCC cell lines of SCC25, HN5 and Tca8113 were kindly supplied by Associate Professor Nick Saunders (The University of Queensland, Australia), Professor Ming Wei (Griffith University, Australia) and Professor Qian Tao (Sun Yat-sen University, China) respectively. These cells were routinely maintained in DMEM containing 10% FBS. To examine the effects of TGF-β1, equal number of these cells were plated in
serum-free medium, starved for 12h, and then treated with TGF-β1 (5 ng/mL) for 0-3 days. Culture medium was changed every day.

4.3.2 Cell proliferation assay

OSCC cells were seeded at a density of 5×10^3 cells/well in 96-well plates, allowed to attach overnight and then treated as above. Absorbance was read at 590 nm on a Biomek plate reader (Beckman Coulter, Gladesville, Australia), after addition of 20 μL methylthiazol tetrazolium (MTT, 5 mg/mL, Life Technologies) to the wells for 4h incubation, followed by removal of all solution and addition of 150 μL/well of dimethyl sulfoxide (DMSO) to solubilise the cells.

4.3.3 Immunocytochemistry

After the treatment with TGF-β1 for 0-3 days, OSCC cells were fixed with 10% formalin for 10 min and permeabilized by 0.1% Triton X-100 for 5 min. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide for 10 min. Non-specific binding of the antibodies was avoided by blocking with 5% BSA in PBS for 30 min. Thereafter, the fixed cells were incubated overnight at 4°C with the primary antibodies of CK (1:50) and VIM (1:50), and then with the secondary antibodies for 1h at 37°C. Non-immune serum instead of the primary antibody was used as negative controls. Three washes with PBS were applied between each step of antibody incubation. Sites of binding were visualized by using liquid diaminobenzidine (DAB) + substrate + chromogen system, counterstained with Mayer’s haematoxylin, and photographed by a Nikon OXMA1200 digital camera with the Act-1 program.
4.3.4 Real-time PCR

Total RNA was isolated from OSCC cells before and after TGF-β1 treatment using the PureLink RNA Mini Kit (Life Technologies, USA). RNA was reverse transcribed to cDNA by utilising the iScript cDNA Synthesis Kit (Bio-Rad, USA) based on the manufacturer’s instructions. Quantitative gene analysis was performed for Twist1, Snail1, E-cad, N-cad, MMP-2, MMP-9, TIMP-1, TIMP-2, MT1-MMP, RANKL and OPG by using EXPRESS SYBR GreenER qPCR Supermix Universal Kit (Life Technologies, USA) and the iCycler iQ5 real-time PCR system (Bio-Rad, USA). The data were normalized to the internal control, GAPDH to obtain ΔCt. Finally fold-change of genes of interest relative to untreated samples was reported by \(2^{\Delta\Delta C_t}\) method. Primers used in this study are listed in Table 2.1.

4.3.5 Gelatine zymography

CM from OSCC cells before and after TGF-β1 treatment was used for gelatine zymography. CM of HT 1080 cells served as the positive control. Equivalent amounts of protein per sample, were mixed with non-reducing sample buffer (62.5 mM Tris-HCL, pH 6.8; 4% SDS; 25% glycerol; 0.01% Bromophenol Blue) and electrophoresed on 10% precast denaturing SDS polyacrylamide gels with gelatine (Bio-Rad Lab, CA, USA). Gels were washed in the commercial renature solution (2.5% Triton X-100) for 40 min at room temperature and incubated in development solution (50 mM Tris; 200 mM NaCl; 5 mM CaCl₂, 0.02% Brij-35) at 37°C for 40 h. Finally, gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, USA) for 1
h at room temperature and progressively destained until clear bands appeared against the blue background.

4.3.6 Western blotting

Total protein was extracted from OSCC cells before and after TGF-β1 treatment using radioimmunoprecipitation lysis buffer (Thermo Scientific, USA). The protein concentration was determined by using a BCA Protein Assay Kit (Thermo Scientific, USA). 40 μg of protein was subjected to SDS–PAGE with 10% polyacrylamide gels. Proteins were transferred to PVDF membranes, and then blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature. The membranes were then incubated with primary antibodies of Twist1 (1:200), Snail1 (1:200), E-cad (1:200), N-cad (1:100), TIMP-1 (1:200), TIMP-2 (1:200), MT1-MMP (1:200), RANKL (1:500), OPG (1:200) and α-Tubulin (1:3000) overnight at 4°C, washed twice and incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies for 1 h at room temperature. The protein bands were detected by SuperSignal WestPico Chemiluminescent Substrate (Thermo scientific, USA) and visualised using the VersaDoc-MP Imaging Systems (Bio-Rad).

4.3.7 Osteoclast differentiation from RAW264.7 cells

Cells of RAW264.7 were cultured in DMEM with 10% FBS at 37°C in a humidified atmosphere with 5% CO₂. To obtain osteoclasts, these cells were seeded in a 96-well plate at a density of 1.25×10⁴ cells/well, and supplemented with 50 ng/mL of recombinant mouse RANKL (R&D, USA) on day 1 and day 3. Mature osteoclasts
were observed on day 5. Afterwards, the entire culture medium was changed into CM of OSCC cells pre-treated with or without TGF-β1 (5 ng/mL). After 4 days' treatment, tartrate-resistant acid phosphatase (TRAP) staining (STrACP, Nanjing Jiancheng) was performed and photographed by using a Nikon OXM1200 digital camera with the Act-1 program.

### 4.3.8 Immunohistochemistry

To validate proteins of CK, VIM, E-cad and Snail1, we examined archival blocks from 12 patients whose OSCC showed invasion of bone. Serial tissue sections (5 μm thickness) were dewaxed, rehydrated and treated with 0.3% hydrogen peroxide in PBS. Antigen retrieval was performed by heating sections in a microwave oven (2×4 min) in 0.2% citrate buffer (pH=6.0). After non-specific binding was blocked with 5% BSA in PBS for 30 min, sections were incubated with primary antibodies of CK (1:50), VIM (1:50), E-cad (1:80) and Snail1 (1:100) overnight at 4°C. Sections were then treated with the anti-mouse/rabbit secondary antibodies (Envision + Systems) for 30 min, followed by dianaminobenzidine (DAB) detection solution (Dako, Botany, Australia) for a few minutes at room temperature. Primary antibodies were replaced by non-immune serum as negative controls. Sections were counterstained with Mayer’s haematoxylin, dehydrated, and mounted with DPX (BDH Laboratory, Poole, England). The final results were visualized by light microscopy and photographed using an Olympus Bx60 digital camera with the CellSens software.

### 4.3.9 Statistical analysis
Data analysis was performed using SAS program (SAS version 8.1, USA). A paired Student t test was used to compare two means. One way analysis of variance (ANOVA) is applied to compare two or more means, followed by Student-Newman-Keuls (S-N-K) test. A p value of less than 0.05 was regarded as significant.

4.4 Results

4.4.1 Previous study of indirect co-culture between osteoblasts and OSCC cells

In our previous studies,\textsuperscript{147} CM was collected from OSCC cell lines SCC15 and SCC25, and from the cell line of human foetal osteoblasts (hFOB), and used for indirect co-culture: OSCC cells were treated with CM of hFOB cells for 48hs. Results showed that Twist1 protein expression was up-regulated after the treatment. MMP-2 protein amount was increased while MMP-9 was decreased in all OSCC cells (Fig.4.1A). Immunochemical staining of these molecules was observed in 12 clinical samples of OSCC patients with bone invasion (Fig.4.1B): H&E staining showed an infiltrative pattern of bone invasion with tumour cells invading the bone, and osteoclasts accumulated in resorption lacunae. Weak staining of Twist1 was found in the cytoplasm of OSCC cells, but more strongly in osteoclasts. MMP-2 was weakly expressed in OSCC cells and osteoclasts, while MMP-9 was clearly localized within the cytoplasm of OSCC cells and especially in the nuclei of osteoclasts.
Figure 4.1. Data from our previous study of the indirect co-culture between OSCC cells and osteoblasts (hFOB).\textsuperscript{147} (A) Western blotting showed that Twist1 protein expression was up-regulated after the treatment. The amount of MMP-2 protein was increased, while MMP-9 was decreased in all OSCC cells. (B) Immunohistochemical staining of these molecules was confirmed in clinical samples of OSCC patients with bone invasion: H&E staining showed an infiltrative pattern of bone invasion, and osteoclasts accumulated in resorption lacunae. Weak staining of Twist1 was found in the cytoplasm of OSCC cells, but more strongly in osteoclasts. MMP-2 was weakly expressed in OSCC cells and osteoclasts, while MMP-9 was clearly localized within the cytoplasm of OSCC cells and especially in the nuclei of osteoclasts.
4.4.2 TGF-β1 had no effect on proliferation or morphology of OSCC cells

MTT results showed that TGF-β1 did not affect cell proliferation of all OSCC cells tested (Fig.4.2A). Moreover, cell morphology of OSCC cells was not changed, most remaining polygonal. In order to further explore the changes of phenotype, immunocytochemical staining of CK and VIM was performed after each day’s treatment. The results showed similar staining of CK and VIM at each time point: the CK staining was not changed in all OSCC cells before and after treatment with TGF-β1; minimal staining of VIM was found for cells of SCC25 and HN5, while VIM was strongly stained in Tca8113 cells before and after the treatment (Fig.4.2C). A summary of the staining results is shown in Fig.4.2B.

4.4.3 mRNA level changes of selected genes after treatment with TGF-β1

Real-time PCR was utilized to examine mRNA level changes of selected genes. The EMT markers, Twist1 and N-cad, were shown to have increased expression, while Snai11 and E-cad were down-regulated in all cells after treatment with TGF-β1 (Fig.4.3A-C). The cell invasion factor MMP-2 was not affected in SCC25 and Tca8113 cells, while it was induced in HN5 cells. Conversely, MMP-9 was unaffected in HN5, while it was up-regulated in SCC25 and Tca8113 cells. Furthermore, TIMP-1 was suppressed in SCC25 and Tca8113 cells, while HN5 showed increased expression. On the other hand, TIMP-2 was induced in SCC25 and Tca8113, but suppressed in HN5 cells. For osteoclast-related molecules, both MT1-MMP and RANKL were induced while OPG was suppressed in all cells.
Figure 4.2. TGF-β1 had no effect on proliferation or morphology of OSCC cells. (A) MTT results of OSCC cells before and after the TGF-β1 treatment. At each time point of the 3 days' culture, cell proliferation (OD values) of these cells was not affected ($p>0.05$). Data shown as mean ± SD of three independent experiments. (B) Summary of the immunohistochemical staining results of CK and VIM in OSCC cells. Results representative of three independent experiments.
Figure 4.2. TGF-β1 had no effect on proliferation or morphology of OSCC cells. (C) Immunocytochemical staining of CK and VIM in OSCC cells. Similar staining patterns were observed at each time point: CK staining was not changed for all OSCC cells before and after TGF-β1 treatment; minimal staining of VIM was found for cells of SCC25 and HN5 after TGF-β1 treatment; while VIM was strongly stained in Tca8113 cells before and after the treatment (arrow, DAB, bar=25μm).
Figure 4.3. Real-time PCR results of OSCC cells before and after treatment with TGF-β1. (A) EMT markers of Twist1 and N-cad were shown to have increased expression, while Snail1 and E-cad was down-regulated in cells of SCC25 after being treated with TGF-β1. The cell invasion factor MMP-2 was not affected while MMP-9 was up-regulated. Moreover, TIMP-1 was suppressed in SCC25 cells, while TIMP-2 was induced. For osteoclast-related molecules, both MT1-MMP and RANKL were induced while OPG was suppressed. Data were shown as mean ± SD of three independent experiments (*, p<0.05, significantly different from untreated samples).
Figure 4.3. Real-time PCR results of OSCC cells before and after treatment with TGF-β1. (B) EMT markers of Twist1 and N-cad were shown to have increased expression, while Snail1 and E-cad was down-regulated in cells of HN5 after being treated with TGF-β1. The cell invasion factor MMP-2 was induced in HN5 cells while MMP-9 was un-affected. Moreover, TIMP-1 was increased in HN5 cells, while TIMP-2 was suppressed. For osteoclast-related molecules, both MT1-MMP and RANKL were induced while OPG was suppressed. Data were shown as mean ± SD of three independent experiments (*, p<0.05, significantly different from untreated samples).
Figure 4.3. Real-time PCR results of OSCC cells before and after treatment with TGF-β1. (C) EMT markers of Twist1 and N-cad were shown to have increased expression, while Snail1 and E-cad was down-regulated in cells of Tca8113 after being treated with TGF-β1. The cell invasion factor MMP-2 was not affected while MMP-9 was up-regulated. Moreover, TIMP-1 was suppressed in Tca8113 cells, while TIMP-2 was induced. For osteoclast-related molecules, both MT1-MMP and RANKL were induced while OPG was suppressed. Data were shown as mean ± SD of three independent experiments (*, p<0.05, significantly different from untreated samples).
4.4.4 Zymogenic activity changes of MMP-2 and MMP-9 after treatment with TGF-β1

To confirm expressions of MMP-2 and MMP-9, gelatine zymography was used to detect the zymogenic activities of these two factors (Fig.4.4A). Results showed that TGF-β1 increased the activities of MMP-9 in SCC25 and Tca8113 cells, while it increased the activities of MMP-2 in HN5 cells (Fig.4.4B).

4.4.5 Protein amount changes of targeted genes after treatment with TGF-β1

Western blotting was performed to validate expressions of TGF-β1 targeted genes at the protein level. Comparing with results of real-time PCR, the expression pattern was almost the same with a few differences (Fig.4.5A-C). For EMT markers, Twist1 was increased while Snail1 was decreased in all OSCC cells. E-cad was slightly suppressed in SCC25 cells while it was dramatically suppressed in HN5 cells, but not detected in Tca8113 cells. N-cad was found to be induced in all cells tested. TIMP-1 was down-regulated and TIMP-2 was un-changed in both SCC25 and Tca8113 cells; while TIMP-1 was un-affected and TIMP-2 was suppressed in HN5 cells. For osteoclast-related molecules, MT1-MMP was up-regulated in all cells tested. Furthermore, RANKL was induced in SCC25 and Tca8113 cells, while it was not detected in HN5 cells. Expressions of OPG were decreased in all OSCC cells.
Figure 4.4. Gelatine zymography results of OSCC cells before and after treatment with TGF-β1. (A-B) TGF-β1 increased the activities of MMP-9 in both SCC25 and Tca8113 cells, while this increased the activities of MMP-2 in HN5 cells. The results are means of three independent experiments.
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**Figure 4.5.** Western blotting analysis of OSCC cells before and after treatment with TGF-β1. (A-C) For EMT markers, Twist1 expression was increased while Snail1 was decreased in all these cells. E-cad was slightly suppressed in SCC25 while it was dramatically suppressed in HN5, but it was not detected in Tca8113 cells. N-cad was found to be induced in all these cells. TIMP-1 was down-regulated and TIMP-2 was un-changed in both SCC25 and Tca8113 cells; while TIMP-1 was un-affected and TIMP-2 was suppressed in HN5 cells. For osteoclast-related molecules, MT1-MMP was up-regulated in all these cells. RANKL was induced in SCC25 and Tca8113 cells, while it was not detected in HN5 cells. Expressions of OPG were decreased in all these cells. These results are representative of three independent experiments.
4.4.6 CM of OSCC cells pre-treated with TGF-β1 prolonged the survival of mature osteoclasts

Mature osteoclasts generated from the murine macrophage cell line, RAW264.7, were treated with CM of OSCC cells with or without TGF-β1. Since our earlier results had shown significant changes of EMT markers on days 1 and 2, CM of OSCC cells were here treated with TGF-β1 for 24h or 48h. Similar results were observed on each day as follows: comparing with CM from OSCC cells without TGF-β1, CM from OSCC cells pre-treated with TGF-β1 prolonged the survival of osteoclasts up to 4 days (Fig.6A-B). Osteoclasts with continual RANKL treatment became apoptotic on day 4, TGF-β1 (5 ng/mL) also induced the apoptosis of mature osteoclasts on day 4 (Fig.4.6A-B).

4.4.7 Validation of EMT related markers in human OSCC tissues with bone invasion

H&E staining of sections of OSCC from 10 patients with bone invasion showed an infiltrative pattern, and epithelial tumour cells invaded the bone tissue (Fig.4.7A). Immunohistochemistry found that CK was strongly stained in the cytoplasm of OSCC cells at the centre of tumour, while less staining was found in the front of bone resorption sites (Fig.4.7B). Conversely, VIM was weakly stained within the centre of tumour while strongly stained by OSCC cells in the front of bone resorption sites (Fig.4.7C). For E-cad, weak cytoplasmic expression was found at the centre of tumour while no staining was shown in the bone resorption sites (Fig.4.7D). Stronger cytoplasmic staining of Snail1 was observed in the cytoplasm of all OSCC cells (Fig.4.7E). Control sections were negative (Fig.4.7F).
Figure 4.6. TRAP staining of the mature osteoclasts generated from RAW264.7 cells. (A) The positive control group of osteoclasts (with continual RANKL treatment) became apoptotic on day 4 (TRAP, bar=25μm). TGF-β1 (5 ng/mL) also induced the apoptosis of mature osteoclasts on day 4 (TRAP, bar=25μm). Comparing with CM of OSCC cells without TGF-β1, CM of OSCC cells pre-treated with TGF-β1 could prolong the survival of mature osteoclasts up to 4 days (arrow, TRAP, bar=25μm). (B) the number of osteoclasts was counted with 4 fields randomly selected. Data are shown as mean ± SD of three independent experiments (*, p<0.05).
Figure 4.7. Immunohistochemical analysis of targeted molecules in OSCC tissue with bone invasion. (A) H&E staining showed the infiltrative pattern of bone invasion by OSCC, and epithelial tumour cells invaded the bone tissue. (B) Immunohistochemistry showed that CK was strongly stained in the cytoplasm of OSCC cells at the centre of tumour, while less staining was found in the front of bone resorption sites. (C) VIM was weakly stained within the centre of tumour while strongly stained by OSCC cells in the front of bone resorption sites. (D) Weak cytoplasmic expression of E-cad was found at the centre of tumour while no staining was shown at the bone resorption sites. (E) Stronger cytoplasmic staining of Snail1 was observed in the cytoplasm of all OSCC cells. (F) Control sections showed negative staining.
4.5 Discussion

In general terms, TGF-β acts as a tumour suppressor to control the proliferation of normal cells. When cell proliferation exceeds normal limits, TGF-β prevents overgrowth through anti-proliferative and pro-apoptotic actions.\textsuperscript{142} But cells which have undergone malignant transformation can antagonise its inhibitory effects and selectively shut down its suppressive arm, due to specific gene mutations, or loss of function of TGF-β signalling components.\textsuperscript{141,142} In our present study, the MTT assay showed that TGF-β had no obvious effect on inhibiting the growth of these OSCC cells. Similar results were also reported by Takayama’s group, namely that TGF-β (at 5 ng/mL) did not inhibit the proliferation of a murine OSCC cell line (SCCVII) with bone invasive properties.\textsuperscript{59} These researchers suggested that proliferative abilities of OSCC cells may not be affected in the initial period of bone invasion.

TGF-β is well known to be a key initiator of EMT, which can induce artificial EMT of normal epithelial cells as well as of malignant cells.\textsuperscript{74,143} The process of EMT is characterized by changes of cell shape, in which epithelial cells become detached from each other, and acquire a more migratory phenotype akin to mesenchymal cells.\textsuperscript{148} A hallmark of EMT is the disintegration of cell-cell junctions, particularly adherens junctions, this dissociation being orchestrated by several transcriptional factors, such as the zinc-finger proteins of Snail/Slug, or basic helix-loop-helix (bHLH) family members of Twist1.\textsuperscript{74,143} In our study, we observed that cell morphology in these OSCC cells cultures was not changed, most cells remaining polygonal during 3 days’ treatment with TGF-β1. This is consistent with our earlier studies in which morphological evidence of EMT took several days longer to become
manifest. In order to explore the changes in phenotype more precisely, therefore, immunohistochemical staining of CK and VIM was carried out. Normally de novo expression of VIM correlates with down-regulation of CK, and these have been proposed as crucial markers of the fibroblastoid state. We found that CK staining was almost the same in cells tested before and after the treatment. Meanwhile, minimal staining of VIM was found in SCC25 and HN5. Interestingly, VIM was strongly stained in the non-treated cultures of Tca8113, suggesting this cell line has special characteristics. VIM has been noted to be present in leukocytes, endothelial cells, and in some carcinomas without signs of dedifferentiation or EMT. The value of VIM expression as a marker of EMT clearly needs further investigation.

For these EMT markers, typical “cadherin switching”, a term referring to the ability of E-cad expression and activity to give way to that of N-cad, was shown in all cell lines tested, although validation at protein level could not detect N-cad in cells of Tca8113. Twist1 expression was increased while Snail1 was decreased in all cells after being treated with TGF-β1. Both Twist1 and Snail1 are nuclear transcription regulators of EMT which interact with each other, not always in a consistent fashion. For example, Twist1 was found to act upstream from Snail and induce EMT-like transformation in a mouse xenograft model with human breast cancer, while a comparison of benign and malignant pheochromocytoma suggested to the authors that Snail may target on Twist to promote malignant transformation. On the other hand, in a comprehensive examination of human hepatocellular carcinoma, Yang et al found that Snail and Twist were independently regulated but worked collaboratively to suppress the transcription of E-cad. Whilst the reasons for these different interpretations arising in different experimental situations are currently unknown, the
present study, consistent with previous co-culture research, and the increased expression of Twist1 which results, suggests that TGF-β1 maybe the actual factor in CM which initiates EMT.

MMP family members are the main proteases responsible for digestion of the extracellular matrix (ECM).\textsuperscript{154} The gelatinases MMP-2 and MMP-9, are not only involved in degrading ECM, but also associate with EMT to increase tumour invasion and metastasis.\textsuperscript{155} We have previously reported that expression of MMP-2 and MMP-9 in OSCC cells was, in relation to EMT, triggered by TGF-β1.\textsuperscript{156} MMP-2 and MMP-9 are secreted as latent zymogens, and rapidly become inhibited by the specific endogenous inhibitors TIMP-1 and TIMP-2.\textsuperscript{156,157} Our study found MMP-2 expression did not alter in response to TGF-β1, but MMP-9 expression increased; TIMP-1 expression decreased and TIMP-2 increased in both SCC25 and Tca8113 cells. Conversely, MMP-2 expression was up-regulated and MMP-9 was not changed, while TIMP-1 expression was up-regulated and TIMP-2 was down-regulated in HN5 cells. The increased amount of MMP-9 or MMP-2 in these OSCC cells suggested similar responses to TGF-β as shown by other researchers.\textsuperscript{158,159} This may establish a positive autocrine loop: both MMP-2 and MMP-9 can mediate the cleavage of latent TGF-β complexes.\textsuperscript{160} Moreover, endogenous regulation of MMP and TIMP was confirmed by the observations that endogenous MMP-9 was inhibited by TIMP-1, and TIMP-2 was the primary inhibitor of MMP-2. The balance between MMP and TIMP was disturbed, in favour of increased expression of MMPs, which might directly facilitate infiltration of OSCC cells through soft tissues or marrow spaces within the bone.
Osteoclast-related factors not only include molecules which induce the formation of osteoclasts, but also those proteolytic enzymes which help to degrade the non-mineralized osteoid. They function by reinforcing malignant keratinocytes to break down matrix components, and act on bone cells to control their differentiation and maturation. The gelatinases MMP-2 and MMP-9 participate in the recruitment of osteoclast precursors and the differentiation of osteoclasts during the development and growth of normal tissues. In addition to the gelatinase functions of MMP-2 and MMP-9, MT1-MMP functions as a sheddase, releasing non-ECM substrates such as RANKL. A recent report also demonstrated that MT1-MMP derived from prostate cancer cells enhanced their migration through an autocrine pathway of MT1-MMP/RANK/RANKL. The present study has shown that MT1-MMP was increased in all OSCC cells tested. To establish whether MT1-MMP has similar autocrine effects in OSCC cells as demonstrated for prostate cancer cells, we analysed the expression of RANKL and OPG, which have been proven to be important in osteoclast activation and subsequent bone destruction. We found the expression of RANKL was increased while OPG was decreased in all OSCC cells. Therefore, the suppression of OPG expression tipped the ratio of RANKL to OPG in favour of RANKL, and this may lead to enhanced osteoclastogenesis and increased bone resorption.

We next wished to investigate the effects of molecules released by OSCC cells on osteoclast behaviour (Pathway C, Figure 4.8). Unexpectedly, when we added CM of OSCC cells into cultures of RAW264.7 cells, we did not observe osteoclast formation (data not shown). Thereafter, we firstly obtained mature osteoclasts from RAW264.7 cells, and treated them with CM of OSCC cells grown with or without TGF-β1. Our
results showed that CM from cells pre-treated with TGF-β1 prolonged osteoclast survival up to 4 days compared with CM without TGF-β1. With continued RANKL treatment, mature osteoclasts became apoptotic on day 4. TGF-β1 (5 ng/mL) also induced apoptosis of these osteoclasts. Our results indicated that factors in OSCC cells treated by TGF-β1 might block its apoptotic effects, and promote survival of osteoclasts.165

The question of whether changes in the shape of neoplastic cells are necessary for these to acquire osteomimetic characteristics remains. Some researchers in the field of bone metastasis have proposed that malignant epithelial cells have to become fibroblast-like, before they are able to express osteogenic factors.77-79,166 Davies et al found that transfection of TGF-β1 into a rat keratinoocyte cell line caused changes of cell morphology from polygonal to spindle; these spindle cells subsequently formed tumours in nude mice, with higher incidence of local bone resorption.75 Takayama et al reported that TGF-β1 caused EMT of SCCVII cells, and they found EMT-like changes through resected human mandibles with gingival SCC, that immunohistochemical staining of E-cad was weak in tumour margins comparing with central lesions.59 In our present study, although TGF-β1 did not induce the full changes of cell morphology indicative of EMT, these OSCC cells could maintain the survival of osteoclasts. Further validation of molecules in OSCC tissue samples found stronger staining of CK and Snail1, while weaker staining of VIM and E-cad, which are consistent with Takayama’s results. Although these findings are indirect observations, which are not substantial, they suggest that partial EMT may occur during the progressive invasion of bone by OSCC. Whether morphologic and
phenotypic changes of malignant keratinocytes are necessary for bone invasion is the focus of future work.

To sum up, our studies have shown that EMT—perhaps only partial EMT—of OSCC cells, triggered by TGF-β1, may be associated with bone invasion by OSCC. TGF-β1 may not only induce EMT which thus increases the invasive capacity of OSCC cells, but also promotes expression of osteoclastic factors and prolongs osteoclast survival (Figure 4.8). These findings may provide insights into the underlying mechanisms of bone invasion by OSCC, and ultimately provide molecular information for developing targeted therapeutics.

**Figure 4.8.** Pathways involved in the progression of bone invasion by OSCC. (A) Osteoclasts play the main role to degrade the bone matrix, growth factors such as TGF-β are released from the bone components. (B) TGF-β may induce EMT of OSCC cells and promote expressions of MMPs, which directly facilitate infiltration of more tumour cells through soft tissues or marrow spaces within the bone. (C) TGF-β may also promote cytokines such as osteoclasts-related factors, which activate more osteoclasts and prolong their survival, again favouring invasion.
Chapter 5

The MCP-1 gene as a potential target to inhibit the bone invasion by OSCC
5.1 Introduction

With the breakthrough of biological target therapy, the treatment options for patients with bone destruction caused by cancer have been greatly expanded. A variety of treatments can be chosen now and biotherapy targeting on osteoclasts is a rational approach to reduce the risk for skeletal complications. Since OSCC has the properties of bone invasion and osteoclasts play an important role in its progression, finding an efficient target associated with osteoclasts for biotherapy is essential, which may enhance the therapeutic approaches in clinical management.

Chemokines (chemotactic cytokines) are a large family of small heparin-binding peptides, generally 70 to 90 amino acids in length. According to the number and arrangement of serine residues, chemokines are segregated into four subfamilies: CXC, CC, C, and CX3C - , which have similar functions in terms of activation of leucocytes through their binding to selective receptors coupled with G-protein. To date, more than 50 chemokines and 20 chemokine receptors have been identified, which are known to play a crucial part in homing leukocytes and other cells in the human body, engendering both physiological and pathological processes.

Monocyte chemoattractant protein-1 (MCP-1), also termed as chemokine (C-C motif) ligand 2 (CCL2), is one of the key members of CC chemokine family, which functions as a potent agonist for monocytes, dendritic cells, memory T cells and basophils. Note that MCP-1 is among the most thoroughly characterized members of the chemokine family; it has been shown to be a potential intervention point for the treatment of various diseases, such as multiple sclerosis, atherosclerosis and
rheumatoid arthritis.\textsuperscript{174} Moreover, MCP-1 has been found to be over-expressed in several types of malignancy including myeloma, breast or prostate cancer, and can mediate the osteoclastogenesis induced by neoplasms.\textsuperscript{175-177} For example, using a xenograft model in SCID mice, Mizutani et al reported that overexpression of MCP-1 in the prostate cancer cell line, PC-3, enhanced the growth of bone metastases.\textsuperscript{178} Histological analysis showed that increased numbers of functional osteoclasts were found in bone samples. To date, however, we can find no report concerning possible roles of MCP-1 in the invasion of bone by OSCC.

The progression of bone invasion by OSCC is a highly coordinated process, and may be divided into three phases: initial, resorption and final phases.\textsuperscript{143} The main stage is the resorption phase, where osteoclasts play the key roles to degrade bone matrix. Cytokines generated from malignant keratinocytes may directly or indirectly induce the formation of osteoclasts. Active osteoclasts are frequently observed in clinical tissue samples from OSCC patients with bone invasion, which usually appear in the edge of bone tissue and sit close to the neoplastic cells themselves. This phenomenon raises a specific question about how these osteoclasts are recruited or mobilized to the resorption sites. It also reminds us of the potential therapeutic value of blocking the messages which pass between malignant epithelial cells and osteoclasts, which may inhibit the progression of bone invasion by OSCC.

In addition to its expression in cancer cells, MCP-1 has also been identified as one of the typical chemokines associated with development and function of osteoclasts. Kim et al reported that MCP-1 is induced by RANKL, where it accelerates the differentiation of human osteoclasts in an autocrine manner.\textsuperscript{179} Furthermore, human
peripheral blood mononuclear cells (PBMCs) treated by MCP-1 formed TRAP positive multinucleated osteoclast-like cells, but those cells were unable to resorb bone, indicating that MCP-1 might promote osteoclast fusion, which is a key event of osteoclast differentiation.\textsuperscript{127,130} With these reports, the aim of our present study is to investigate whether MCP-1 can be used to inhibit bone invasion by OSCC via targeting on osteoclasts. We used the vector of 7ND,\textsuperscript{174} a dominant-negative inhibitor of MCP-1, to transfet the OSCC cell line SCC25, and sought evidence of inhibiting effects both, \textit{in vitro} and \textit{in vivo}.

5.2 Materials

DMEM, Opti-MEM, foetal bovine serum (FBS), trypsin-EDTA, G418, and Alexa Fluor 594 (PE) conjugated secondary antibody were purchased from Life Technologies (USA). \(\alpha\)-MEM was obtained from Hyclone (Thermo Scientific, USA). The primary polyclone antibody of rabbit anti-human MCP-1 was obtained from Abcam (USA), the other primary antibody of rat anti-mouse CD14 was obtained from eBioscience (USA). The transfection reagent of FuGENE HD was got from Promega (USA). Recombinant human cytokines of CSF1 and RANKL were purchased from Peprotech (Rocky Hill, NJ, USA).

5.3 Methods

5.3.1 Immunohistochemistry

To detect the expression of MCP-1 protein, 10 archival blocks were examined from 10 patients whose OSCC showed invasion of bone, following the approval of
Australian Capital Territory (ACT) Heath Human Research Ethics Committee. Serial tissue sections (5 μm thickness) were dewaxed, rehydrated and treated with 0.3% hydrogen peroxide in PBS. Antigen retrieval was performed by heating sections in a microwave oven (2×4 min) in 0.2% citrate buffer (pH=6.0). After non-specific binding was blocked with 5% BSA in PBS for 30 min, sections were incubated with the primary antibody of MCP-1 (1:80) overnight at 4°C. Sections were then treated with the anti-rabbit secondary antibody (Envision™ + Systems) for 30 min, followed by diaminobenzidine (DAB) detection solution (Dako, Botany, Australia) for a few minutes at room temperature. The primary antibody was replaced by non-immune serum as negative controls. Sections were counterstained with Mayer's haematoxylin (Sigma, USA), dehydrated, and mounted with DPX (BDH Laboratory, Poole, England). The final results were visualized by light microscopy (Leitz Laborlux S, Germany) and photographed using a Olympus BX60 camera with CellSens software (Olympus, Japan).

5.3.2 Cell lines and culture conditions

Normal dermal epithelial cells (EP) were kindly supplied by Queensland Institute of Medical Research (QIMR, QLD, Australia). The prostate cancer cell line PC3 was supplied by Professor Jin Gao (James Cook University, QLD, Australia). The OSCC cell line SCC25 was a gift of Associate Professor Nick Saunders (Princess Alexandra Hospital, QLD, Australia). The OSCC cell lines HN5 and Tca8113 were kindly given by Professor Ming Wei (Griffith University, Australia) and Professor Qian Tao (Sun Yat-sen University, China) respectively. All cells were maintained in DMEM
supplemented with 10% FBS and antibiotics (100 U/mL of penicillin G and 100 mg/mL of streptomycin, Invitrogen) at 37°C in an incubator (5% CO₂/20% O₂).

5.3.3 Real-time PCR

At the same time point, all cells were plated in 6-well plates at a density of 1x10⁶ cells per well. After an overnight culture and when cells attained confluence, total RNA from each cell line was isolated using the PureLink RNA Mini Kit (Life Technologies, USA). RNA was quantified and quality tested by spectrophotometry and gel electrophoresis. All samples were confirmed at 260/280 absorbance ratios between 1.8 and 2.1 and had prominent 18s and 28s bands. Thereafter, total RNA was converted into cDNA using ImProm-II Reverse Transcriptase (RT; Promega) and oligo dT primer (Promega). Quantitative expressions of MCP-1 and 18s ribosomal RNA (rRNA) were performed using EXPRESS SYBR GreenER qPCR Supermix Universal Kit (Life Technologies, USA). The primer sequences used to amplify 18s rRNA were 5’-CTTAGAGGGACAAGTGCGG-3’ and 5’-ACGCTGAGCCAGTCAGTGTA-3’; MCP-1 were 5’-TCGCGAGCTATAGAAGAATCA-3’ and 5’-TGTCAAGTCTTCCGAGTTG-3’; Thermal cycling was started at 95°C for 2.5 min, followed by 45 cycles of amplification at 95°C for 10 sec, 58°C for 10 sec, 72°C for 25 sec and 72 cycles of elongation at 60°C for 5 sec as the final step. All reactions were carried out on the iCycler iQ5 real-time PCR system (Bio-Rad, USA). The data were normalized to the internal control, 18s rRNA, to obtain ΔCt. The relative quantification of mRNA expression of MCP-1 gene was reported by the 2⁻ΔΔCt method.
5.3.4 Cell transfection

The 7ND expression vector (7ND) was kindly supplied by Associate Professor Kensuke Egashira (Kyushu University, Japan). FuGENE HD was used to mix 7ND, and the ratio between them was set as 1:3 (v/v) based on the optimization of previous studies. Briefly, cells of SCC25 were seeded into a 24-well plate at a density of $5 \times 10^4$ cells/well, with 500 µl of complete medium. After 24h, the cells were ready for transfection and complete medium was changed into Opti-MEM. Different amounts of 7ND (0.2 µg, 0.4 µg, 0.6 µg) were added to Opti-MEM respectively, to which the required amount of FuGENE HD was added to a final volume of 20 µL. This 7ND-FuGENE mixture was incubated for 15 min at room temperature to allow the transfection complex to form. Then the transfection complex was added dropwise to the cells. After 48h, Opti-MEM was changed into complete medium and antibiotic selection was started.

5.3.5 Establishing stabilized SCC25 cells with 7ND vector by antibiotic selection

To establish a stably transfected OSCC cell line with 7ND vector, the transiently transfected cells of SCC25 was placed under G418 antibiotic selection. Before doing this, the G418 kill curve assay was performed to determine the optimal concentration of G418 at which 100% cell death would be observed in normal SCC25 cells. For SCC25 cells transfected with different amounts of 7ND (0.2 µg, 0.4 µg, 0.6 µg) after 24 hours, G418 treatment commenced at this concentration. After approximately 6 days, the cultures were split in half and replated into 24-well plates. Antibiotic administration continued for another three weeks, at which time net growth was
observed. Thereafter, the cells from three of the most resistant cultures were removed from the 24-well plate, pooled and then re-seeded by a one in two serial dilution in duplicate over two 24-well plates. After 4 days, these cells growing from a single colony were transferred to a new well in a 12-well plate. The cells were up-scaled further until they were grown in 25 cm² tissue culture flasks. These cells were then lysed, total RNA was extracted, and cDNA was subsequently synthesized. Real-time PCR was used to determine the relative gene expression of MCP-1 in all these cells, that with the greatest level (SCC25-7ND) being used in the following studies.

5.3.6 CD14⁺ monocyte separation and the indirect cell co-culture

Peripheral blood mononuclear cells (PBMCs) were isolated from human blood collected into BD vacutainer cell preparation tubes (CPT) with sodium citrate (BD, USA). After centrifugation at 1500 g for 30 min, the cell layer on top of the Ficoll-Paque in the CPT was collected, resuspended in 10 ml of α-MEM and re-centrifuged (1250 rpm, 10 min). Pure CD14⁺ monocytes were extracted from PBMC by incubation with MACS CD14 human microbeads (Miltenyi Biotec, Bisley, UK) for 15 min at 4°C. Cells were washed by using CD14⁺ isolation buffer (0.5% FCS; 2 mM EDTA, pH 8) and passed through an autoMACS magnetic cell separator (Miltenyi Biotec, Bisley, UK). Then these CD14⁺ monocytes was planted in 24-well plates at 1×10⁵ cells per well in 600 µL of medium (α-MEM, pH 7.4, containing 10% FBS and 1% penicillin/streptomycin), with the recombinant human cytokines CSF1 (25 ng/mL) and RANKL (40 ng/mL) for generating the osteoclasts.
The indirect cell co-culture of CD14⁺ monocytes and SCC25 cells was performed in the 24-well culture plate, and groups were arranged as follows: Group 1. CD14⁺ monocytes only; Group 2. CD14⁺ monocytes with CSF1 (25 ng/mL) and RANKL (40 ng/mL); Group 3. CD14⁺ monocytes with CSF1 (25 ng/mL) and RANKL (40 ng/mL), plus 10% (v/v) condition medium (CM) of SCC25 cells; Group 4. CD14⁺ monocytes with CSF1 (25 ng/mL) and RANKL (40 ng/mL), plus 10% (v/v) CM of SCC25-7ND cells. CM (supernatant) was collected as follows: cells were washed with phosphate buffered saline (PBS) and then the medium was changed into α-MEM without FBS. After 48h culture, the entire medium was collected and centrifuged at 1,500 g, for 20 min at 4°C to remove any cell debris. After these monocytes had been cultured under the same condition for 7 days, osteoclasts were formed: these were subsequently fixed in 10% formaldehyde solution and stained for TRAP. TRAP⁺ cells that had three or more nuclei were considered to be multinuclear osteoclasts. Hoechst staining (Cellomics, Pittsburgh, PA) was used to visualize nuclei. Rhodamine-conjugated phalloidin (Life Technologies, USA) was used to stain for F-actin.

5.3.7 In vivo model of bone invasion by OSCC

Balb-c nude mice were purchased from the Animal Resources Centre (ARC, Australia). These were housed in the animal facility of Griffith University Gold Coast Campus, and cared by the animal house staff. All protocols were reviewed and approved by Griffith University Ethics Committee (MSC/06/11/AEC). At 6-7 weeks of age, these mice were used to develop a model of bone invasion by OSCC in vivo. Under sterile conditions, OSCC cells (6×10⁶/100 μL) were injected subcutaneously overlaying the calvaria. Mice were randomly divided into three groups (n=6/group):
the negative control group received PBS (Group 1); the positive control group received cells of SCC25 (Group 2); the experimental group received cells of SCC25-7ND (Group 3). All animals were sacrificed after 6 weeks. Tumours and calvarias were surgically removed and fixed in 4% paraformaldehyde (PFA, Sigma) for histological and immunohistochemical analysis.

5.3.8 Flow cytometry analysis

Bone marrow cells (BMCs) from mice in each group were taken out from tibia on Week 6. The CD14 subpopulation of BMCs were evaluated by incubating $1 \times 10^6$ cells with the anti-CD14 antibody in PBS at 4°C for 30 min. Thereafter, cells were stained with PE-conjugated rat anti-mouse secondary antibody. Flow cytometry analysis (FACS) was carried out on a FACScan flow cytometer (BD, USA). The unstained cells were gated out and data acquisition with analysis was performed using CellQuest software (BD, USA).

5.3.9 Histologic and immunohistochemical analyses

PFA-fixed tumour samples collected from these nude mice in different groups were processed for paraffin sectioning. Serial 5 μm sections was cut on Leica RM 2235 rotary microtome (Leica Microsystems, Canada) and stained with haematoxylin and eosin (H&E). Immunohistochemical staining of the sections was performed by incubation of serial sections with the primary antibody of MCP-1 (1:80) overnight followed by HRP labelled secondary antibody and DAB staining. Specimens treated with non-immune serum served as control. To perform histochemical staining for
these tumour-bearing calvaria, all calvarias were decalcified in 10% EDTA (pH=7.4) for 2 weeks and processed for paraffin embedding. Serial 5 μm sections of paraffin embedded calvaria were stained by both H&E and TRAP. Histomorphometric analysis of TRAP-positive osteoclast numbers at the tumour-bone interface was performed. For each section, an area of 2 mm² with the tumour-bone interface was defined for counting osteoclast numbers. Four fields of this area were randomly selected and counted to determine the numbers of TRAP-positive osteoclasts.

5.3.0 Statistical analysis

Data analysis was performed using the SPSS software (SPSS 20.0, IBM, USA). Student t test was used to compare two means. One way analysis of variance (ANOVA) is applied to compare two or more means, followed by Student-Newman-Keuls (S-N-K) test. A p value of less than 0.05 was regarded as significant.

5.4 Results

5.4.1 Strong staining of MCP-1 protein was found in tissue samples from OSCC patients with bone invasion

Sections of OSCC samples from 10 patients with bone invasion were investigated for the expression of MCP-1. IHC showed that MCP-1 protein was strongly stained in osteoclasts and tumour cells (Fig.5.1A). Control sections were negative. A summary of the staining results is shown in Fig.5.1B.
Figure 5.1. Staining of MCP-1 protein in OSCC tissue samples with bone invasion. (A) MCP-1 protein was strongly stained in osteoclasts and in tumour cells. A control section (lower right) is negative. (B) Summary of the staining results of all 10 samples.

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<th>Case</th>
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<th>Staining Density</th>
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<td>3</td>
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5.4.2 MCP-1 mRNA was highly expressed in 3 cell lines of OSCC

Real-time PCR showed that MCP-1 mRNA was highly expressed by 3 different cell lines of OSCC (SCC25, HN5, Tca8113), the cell line SCC25 having the highest expression, which become the candidate cells for cell transfection (Fig.5.2).

A

Marker  EP  SCC25  HN5  Tca8113  PC3

B

Expression of MCP-1 mRNA

Figure 5.2. Expression of MCP-1 mRNA in cell lines of OSCC (SCC25, HN5, and Tca8113). (A) mRNA integrity of all target cells. (B) The cell line SCC25 had the highest expression of MCP-1 mRNA. EP cells served as the negative control. PC3 cells were used as the positive control.
5.4.3 Stabilized SCC25 cells with 7ND vector were generated after antibiotic selection

The 7ND vector was engineered by inserting 7ND sequence into the pcDNA3 vector (Invitrogen), where it was ligated into the BamHI and NotI restriction sites within the polylinker region (Fig.5.3A). It also contains a G418/neomycin resistance cassette for antibiotic selection. The G418 kill curve assay was performed before cell transfection started, and 0.3 mg/mL of G418 was found to be the minimum concentration which caused total cell death of SCC25 cells in 6 days (data not shown). Therefore, 3 concentrations of 7ND vector (0.2 µg, 0.4 µg, 0.6 µg) was used to transfect SCC25 cells, and stabilized cells with 7ND vector were selected by 0.3 mg/mL of G418. Real-time PCR was used to confirm the expression of MCP-1 in these stabilized cells. Results showed that SCC25 cells with 0.6 µg of 7ND vector (SCC25-7ND) had the highest expression of MCP-1 (Fig.5.3C).

5.4.4 10% CM of SCC25-7ND cells efficiently inhibited the formation of human osteoclasts obtained from CD14⁺ monocyte subpopulation

The CD14⁺ monocyte subpopulation was extracted from PBMCs using CD14 microbeads, and osteoclasts were grown from these monocytes with cytokines of CSF1 and RANKL (Fig.5.4A). TRAP staining showed fewer osteoclasts were found when treated with 10% CM of SCC25-7ND cells, compared to 10% CM of SCC25 cells. Immunofluorescence suggested similar results, and less F-actin was observed in those osteoclasts treated with 10% CM of SCC25-7ND cells (Fig.5.4A-B).
Figure 5.3. The 7ND vector was transfected into SCC25 cells and a stabilized cell line was constructed. (A) The structure of the 7ND vector. (B) mRNA integrity of SCC25 cells transfected with different concentrations of 7ND vector (0.2 μg, 0.4 μg, 0.6 μg). (C) Real-time PCR was used to confirm the expression of MCP-1 mRNA in stabilized cells: SCC25 cells with 0.6 μg of 7ND vector (SCC25-7ND) had the highest expression of MCP-1 mRNA.
**A  TRAP staining**

CD14<sup>+</sup> monocytes  

CD14<sup>+</sup> monocytes with CSF and RANKL

CD14<sup>+</sup> monocytes  

CD14<sup>+</sup> monocytes with CSF and RANKL plus 10% CM of SCC25

Figure 5.4. 10% CM of SCC25-7ND cells efficiently inhibited the formation of human osteoclasts generated from CD14<sup>+</sup> monocyte subpopulation. (A) TRAP staining showed less osteoclasts were found in the group treated with 10% CM (v/v) of SCC25-7ND cells.
Figure 5.4. 10% CM of SCC25-7ND cells efficiently inhibited the formation of human osteoclasts generated from CD14⁺ monocyte subpopulation. (B) Immunofluorescence suggested similar results, and less F-actin was observed in the group treated with 10% CM (v/v) of SCC25-7ND cells.
5.4.5 The animal model of bone invasion by OSCC was established by injecting tumour cells onto the surface of calvaria of nude mice

A trial with 3 nude mice was carried out to confirm the injection sites. Cells of SCC25 were injected onto the surface of calvaria where macroscopic tumours formed within one week (Fig.5.5A, photos taken on Week 2). H&E staining of paraffin sections of these tumours showed well-differentiated SCCs (Fig.5.5B), invading the adjacent bone tissue (Fig.5.5C). Numerous osteoclasts were stained by TRAP and found at the tumour-bone interface (Fig.5.5D). Therefore, the animal model of bone invasion by OSCC was established and applied in the formal animal experiments.

5.4.6 The average tumour volume of the SCC25-7ND group was slightly bigger than the SCC25 group

Nude mice of 6-7 weeks were randomly divided into 3 groups (n=6/group): Group 1 received PBS injection; Group 2 received SCC25 cells; Group 3 received SCC25-7ND cells. The final tumour volume (width × length × depth) was recorded on Week 6, and data showed the average tumour volume of SCC25-7ND group was slightly bigger than SCC25 group (Fig.5.6A-B). Significant differences of the tumour volume were found between these groups (p<0.05).
Figure 5.5. Establishment of the animal model of bone invasion by OSCC cells. (A) Tumour formed in one week, localized in the area of calvaria (photos taken on Week 2 of tumour development). (B-C) H&E staining showed well-differentiated OSCC was grown and invaded the adjacent bone tissue. (D) Numerous osteoclasts were stained by TRAP and found in the tumour-bone interface.
Figure 5.6. Comparisons of the tumour volume between different groups of nude mice. (A) Representative photos of tumour formation were taken on Week 6. (B) Data showed the average tumour volume of SCC25-7ND group was slightly bigger than SCC25 group. PBS injection served as negative control (*, $p<0.05$, comparing with PBS group; **, $p<0.05$, significant differences of the tumour volume were found between groups of SCC25 and SCC25-7ND).
5.4.7 The CD14\(^+\) subpopulation of BMCs was reduced in mice injected with cells of SCC25-7ND

FACS analysis showed that the CD14\(^+\) subpopulation of BMCs was slightly reduced in mice injected with SCC25-7ND cells (8.1%, Fig.5.7), in comparison to those mice who received injection with SCC25 cells (9.7%, Fig.5.7). This difference is statistically significant \((p<0.05)\).

Figure 5.7. FACS analysis of the CD14\(^+\) subpopulation of BMCs in nude mice. Results showed that CD14\(^+\) subpopulation of BMCs was slightly reduced in mice injected with SCC25-7ND cells (8.1%), comparing with mice received injection with SCC25 cells (9.7%).
5.4.8 MCP-1 protein was mainly located in the cytoplasm of tumour cells for both groups.

H&E staining showed that well-differentiated SCC formed in both groups, after injection with SCC25 cells and SCC25-7ND cells (Fig.5.8). IHC was performed to locate the MCP-1 protein. This was mostly found in the neoplastic cells, mainly located in the cytoplasm and cell membrane, while less staining was found in nuclei (Fig.5.8).

5.4.9 No significant differences were found between two groups for the numbers of osteoclasts accumulating in the bone resorption lacunae.

H&E staining of both groups showed squamous epithelial OSCC cells invading bone, with many osteoclasts accumulating in typical resorption lacunae (Fig.5.9A). TRAP staining was used to locate and count osteoclasts. There were no significant differences between these two groups for osteoclast numbers (Fig.5.9B).
Figure 5.8. Staining of MCP-1 protein in tumour cells of both groups. H&E staining showed well-differentiated squamous cell carcinoma (SCC) was formed in both groups, after injection of both SCC25 or SCC25-7ND cells. IHC further confirmed that MCP-1 protein was mainly located in cytoplasm and membrane of OSCC cells.
Figure 5.9. The number of osteoclasts in tumour-bone interface. (A) H&E staining of both groups showed OSCC cells invaded the bone tissue, with many osteoclasts accumulating in typical resorption lacunae. TRAP staining was used to locate and count the osteoclasts of all samples. (B) Results suggested there were no significant differences between these two groups for the number of osteoclasts ($p>0.05$).
5.5 Discussion

The development of cancer is associated with the recruitment and infiltration of leukocytes, which contribute an essential element and may facilitate the aggressiveness and distant metastasis of a given neoplasm. Chemokines play an important role in leukocyte homeostasis, which traffick into and out of the tumour microenvironment. Additionally, malignant cells usually express various chemokines and gain functional chemokine receptors. The progression of OSCC is related to the expression of chemokines, for example, the concentration of CXCL8, CXCL10 and CCL14 were found to be significantly elevated in oral fluids of patients with OSCC, comparing with patients of periodontitis, suggesting the chemokine profile of OSCC patients might be a useful parameter in clinical practice. MCP-1, a major attractant of leukocytes, was produced by various cell lines of OSCC in vitro, and increased in oral fluids and tissues from patients with OSCC in vivo. Bektaş-Kayhan et al observed that patients carrying the G allele (GG+AG genotypes) of MCP-1 had a 1.89-fold increased risk of OSCC, in comparison to healthy people. However, little is known as to whether or not MCP-1 plays a critical role in the progression of bone invasion by OSCC. A recent study reported that patients with nasopharyngeal carcinoma with extensive invasion of the skull base had higher concentrations of CCL2 and TNF-α in serum than those without, or with only small invasion, indicating the potential value of MCP-1 as a target for inhibiting bone invasion.

To the best of our knowledge, this is the first report providing information concerning the relationship between MCP-1 and bone invasion by OSCC. In the present study, MCP-1 protein was firstly examined in human tissue samples using IHC, which
showed that MCP-1 was strongly stained in both osteoclasts and tumour cells. The expression of MCP-1 mRNA was detected in 3 different cell lines of OSCC, all of which expressed MCP-1 highly, especially the cell line SCC25. In our previous study, SCC25 cells were also found to differ from SCC15, in that they have faster proliferation. It was reported that 0.23% of side population (SP) cells could be isolated from SCC25 cultures: these possess the characteristics of cancer stem cells, such as higher proliferation rates and colony formation ability, indicating their invasive potential. Taking these observations into consideration, and given the high expression of MCP-1 in SCC25 cells, we selected these for the subsequent inhibitory studies.

7ND is an N-terminal deletion mutant of MCP-1, which lacks amino acids 2-8 and acts as a dominant-negative inhibitor of MCP-1. Specifically, 7ND and wild-type MCP-1 form a heterodimer, which binds to the MCP-1 receptor (CCR2) and completely inhibits the monocyte chemotaxis mediated by MCP-1 in vitro. Furthermore, transgenic mice expressing the gene of 7ND were found to block CCR2 pathway in vivo. Thus, it is possible to use 7ND like an antibody to block the expression of MCP-1 in gene therapy. For example, transfection of 7ND into skeletal muscle is an established strategy for gene therapy of anti-MCP-1. Cells infected with 7ND secrete 7ND protein into circulating blood, and then 7ND protein binds to the MCP-1 receptor on monocytes or target cells in remote organs, thus blocking the signal of MCP-1. Such blockade of endogenous MCP-1 activity was also reported to inhibit the formation of tumour-related vessels and early growth of human malignant melanoma cells grafted in nude mice. Alternatively, mesenchymal stem cells stably transfected with the 7ND gene using lentiviral vector could successfully deliver the
7ND into the lung, which reduced injury caused by bleomycin, and might provide a rationale for patients with acute respiratory distress syndrome (ARDS).  

Based on these reports, for the present study the 7ND vector was utilized to transfet SCC25 cells. Stablized SCC25 cells with the 7ND vector were generated by antibiotic selection with G418. Results of real-time PCR confirmed these SCC25 cells with 0.6 μg of 7ND vector expressed the highest level of MCP-1 mRNA. SCC25-7ND cells are thus a useful tool to perform the following studies, which ask if inhibiting MCP-1 expression can affect the formation of osteoclasts. Since the 7ND vector was secreted into the medium, CM of both SCC25 and SCC25-7ND cells was collected to observe whether these CM could work differently on human osteoclasts.

Thus, monocytes were firstly obtained from PBMCs, and then these CD14+ monocytes were separated using the microbeads. Mononuclear precursors of human osteoclasts are found in the CD14+ monocyte subpopulation, and it has been demonstrated that the expression of CD14 is essential for osteoclastogenesis. Thereafter, an indirect cell co-culture model was set up, with CD14+ monocytes, cytokines of human CSF1 and RANKL, plus 10% CM of SCC25 or SCC25-7ND cells. After 6 days of culture, TRAP staining was applied and showed mature osteoclasts formed in this positive control group with cytokines of human CSF1 and RANKL. For the group with 10% CM of SCC25 cells, many osteoclasts formed, which is consistent with a recent study that, CM of SCC25 could induce the formation of osteoclasts cultured from murine bone marrow cells in vitro. In the group with 10% CM of SCC25-7ND cells, it was observed that fewer osteoclasts formed. Therefore, these results suggest 7ND has been released into the CM and could inhibit
the formation of human osteoclasts \textit{in vitro}. Similar results were also found in previous work of our group, that 7ND significantly suppressed formation of human osteoclasts cultured from PBMCs, and blocked multinucleation, reverting back to mononuclear cell phenotype. The reasons why less F-actin formed in the group with 10\% CM of SCC25-7ND cells are currently unclear, which may be caused by the apoptotic effects of 7ND and needs to be further studied. A recent report described aberrant actin rings in osteoclasts from bone marrow-derived macrophages of MCP-1 knock out mice, which might be related to reduced levels of ERK, Akt, Rac1, and Rho.$^{197}$ There may be other mechanisms by which 7ND inhibits F-actin formation. Also, 7ND may induce CD14$^+$ monocytes to differentiate into other cell phenotypes.

To further prove our hypothesis, an animal model of bone invasion by OSCC was established to check whether 7ND would succeed \textit{in vivo}. This “calvaria-injection” model is attractive for it is easy to inject OSCC cells onto the surface of calvaria of nude mice.$^{198}$ A preliminary study with 3 mice was performed before the formal experiments started. Comparing with other animal models reported,$^{199-201}$ this model has the distinct advantage of causing animal limited stress, as there is no interference with mastication. Through histological studies, it was found that the tumours formed were moderately differentiated SCCs, which invading the bone, with plenty of osteoclasts lining on the edge of resorbed bone. Therefore, the formal experiments were performed with this model, with 3 groups of 6 mice in each group. Group 1 received PBS injection, Group 2 received SCC25 cells, and Group 3 received SCC25-7ND cells. Tumours were formed after 1 week, the final volumes of the resultant tumours being calculated for Groups 2 and 3. The average tumour volume in the SCC25-7ND group was slightly bigger than the SCC25 group. FACS was also used to
compare the CD14 subpopulation of BMCs between these two groups, and showed that this population was reduced in mice with cells of SCC25-7ND. The differences between these two groups are small (8.1% vs 9.7%). This may be because of the small number of animals (n=6) for each group, or because the transfection system leads to limited amounts of 7ND circulating into the bloodstream. However, consistent with previous report of 7ND in vivo,191-193 these results indicate that 7ND vector has circulated in the blood and competed with MCP-1, and this does inhibit monocytes being recruited into the bone marrow.

However, the histological and immunohistological analysis demonstrated there were no significant differences in pattern of bone invasion, nor of the MCP-1 protein expression and bone resorption area between Groups 2 and 3. The tumours formed in both groups showed moderate differentiated OSCC, with similar pattern of bone invasion by OSCC. MCP-1 protein was located mainly in the cytoplasm and membrane of neoplastic cells in both groups. For the bone invasion by OSCC, both groups were observed that tumour cells invading the bone, and many osteoclasts accumulating in the resorption lactune. TRAP staining showed that similar numbers of osteoclasts were found in the bone resorption area of both groups. All these results suggest different mechanisms in vivo as studies in vitro. The reasons why there are no differences between these two groups are currently unknown, and may be explained by several factors. Firstly, the number of animals in each group (n=6) is limited and amplification of the number is necessary in the future studies, which not only increases the sample size but also reduces the variances between the groups. Secondly, the transfection system has yet to be improved, and lentiviral vector or direct injection with the 7ND vector could be used, which may increase the amount of 7ND to
circulate in the blood and target on osteoclasts.\textsuperscript{202} Thirdly, the properties of SCC25 cells may affect the results, since they have not been used for the calvaria-bone research model before, further characterization of their basic properties in this model is necessary.

Another issue of the calvaria-bone model is the lack of immune response, since the animals used are nude mice and immune systems are deficient. Although this model is easily performed for growing tumours which do indeed invade the adjacent bone, this weak point indicates that the model is not totally relevant to the clinical situation. Human OSCCs commonly develop in the tongue, gingiva, and buccal mucosa, and both local and systemic immune responses undoubtedly occur. Immune-deficient mice are utilised in animal models because they are easily established to mimic the invasion and osteolysis of tumour.\textsuperscript{203} Another rodent model of bone invasion by OSCC is to inoculate cells, eg of SCCVII, into the masseter region, were reported in several recent studies.\textsuperscript{59,204,205} This cell line, SCCVII, is derived from an OSCC on the floor of the mouth in mice, and growth of these cells is unaffected by the immune system.\textsuperscript{59,204} For animal work in future, we may generate syngeneic models of OSCC in animals with spontaneous bone invasion, by making cell lines from primary tumours and using these to make animal models of bone invasion. Such syngeneic models could then take full advantage of genetic technologies such as transfection and gene knockouts.

To sum up, the present study has explored roles of MCP-1 in the progression of bone invasion by OSCC. Studies involving transfection of the 7ND vector into SCC25 cells show that competitively inhibiting protein amount of MCP-1 is capable of affecting
osteoclast formation \textit{in vitro}. The function of MCP-1 \textit{in vivo} is also studied, through establishing an animal model of bone invasion by using SCC25 cells with 7ND vector.

All these results demonstrate the relevance of MCP-1 to research on bone invasion by OSCC, and support MCP-1 as a potential target for future gene therapy, as well as therapies of cancer, neuroinflammation and cardiovascular diseases.

Future work will focus on how MCP-1 regulates early differentiation of osteoclasts and the signalling pathways involved, which may help to better understand the progression of this common complication of oral cancer in patients so afflicted.
Chapter 6

Conclusions and Future Directions
This study aimed to investigate the molecular mechanisms of bone invasion by OSCC, and attempted to explore some signalling pathways involved in cross-talk between OSCC cells, osteoblasts and osteoclasts. Bone invasion is a common complication of patients with oral cancer, which severely affects their function of mastication, swallowing, and speech. However, the mechanisms of bone invasion by oral cancer remain unclear. Pathologically, two patterns of bone invasion are recognised: the infiltrative pattern and the erosive pattern. Although the current study mainly focuses on molecular biology to explore potential targets for biological therapies, we also seek to combine the information and correlate molecular findings with pathological patterns, to assist in better understanding the progression of bone invasion by OSCC, improve staging of patients’ disease, and the planning of current surgical approaches.

It is generally thought that invasion of bone by oral cancer is facilitated, enabled or even caused by the activities of osteoclasts, which may be activated by either an indirect pathway involving stimulation of osteoblasts, or directly, initiated by products of the malignant cells themselves. Therefore, in the first part of this thesis, an indirect cell co-culture model was utilized to determine whether CM of osteoblasts or OSCC cells would have effects on each other. We especially focused on MMP-2 and MMP-9, because a previous study from our group had revealed that these two proteins were expressed in malignant keratinocytes, and that the intensity correlated with the presence of lymph node metastasis. Such proteinases will facilitate infiltration of neoplastic cells into soft tissues or bone marrow spaces by degrading the extracellular matrix (ECM). We found that zymogenie activities of both MMP-2 and MMP-9 were increased in OSCC cells after being treated with CM of osteoblasts, while
MMP-9 increased in osteoblasts following culture with CM from OSCC cells. These data indicate their roles in both tumour invasion and osteoclastogenesis. Future studies will focus on the over-expression of MMP-2 and MMP-9 in OSCC cells, and determine what specific roles each one plays in the initial phase of bone invasion by OSCC.

In order to further confirm which part of CM caused the changes of gene expression, TGF-β1, widely reported to be present in significant amounts in the bone microenvironment, was used in the second part of the thesis to treat OSCC cells. Our group had previously found that long term treatment (6 days) of TGF-β1 could induce EMT of OSCC cells in vitro, which encouraged us to study the relationship of such EMT changes and the ability of these cells to invade bone. The theory of EMT is still controversial. Tarin et al have argued that there is no convincing evidence for conversion of epithelial cells into mesenchymal cell lineages in vivo. We also doubt about whether changes of cell morphology are necessary for increased local invasion, since at the advancing front of a carcinoma, tumour cells are usually found in contact with a variety of stromal cells and it is often not possible to differentiate them from mesenchymal cells. In this part of the thesis, it was found the morphology of OSCC cells was not changed after short term treatment with TGF-β1, but partial EMT changes were observed, and CM of these cells with TGF-β1 prolonged the survival of mature osteoclasts generated from cells of RAW264.7. It was surprising that CM of OSCC cells did not induce RAW264.7 cells into osteoclasts. Additional study is needed using these OSCC cells, which have undergone partial EMT, in co-culture with RAW264.7 cells, to study whether direct cell to cell contact, influences further EMT changes and whether the process of osteoclast formation is affected.
To study the indirect pathway of osteoclast recruitment by osteoblasts, we investigated how products of OSCC cells affect osteoclast formation. We focused on MCP-1 in the third part of the thesis, as a previous report from our group had demonstrated that MCP-1 could be strongly induced by RANKL during the formation of osteoclasts.\textsuperscript{179} MCP-1 is also involved in osteoclast differentiation at the stage of multinucleation of osteoclast precursors, and may provide a rationale for increased osteoclastic activities.\textsuperscript{127} Theoretically, if expression of MCP-1 could be blocked in the malignant cells, this may inhibit bone invasion. In this part of the thesis, SCC25 cells were found to have the highest level of MCP-1 mRNA and were used for the subsequent experiments. The inhibitor of 7ND vector was transfected into SCC25 cells, and a stabilized cell line with 7ND vector (SCC25-7ND) was established. Also, 10% CM of SCC25-7ND cells efficiently inhibited the formation of human osteoclasts generated from CD14 subpopulation of monocytes. Although results of the animal experiments differ from the studies of cell lines, the potential value of MCP-1 as an effective target remains. We have shown the delivery by 7ND cell transfection is an efficient way to inhibit MCP-1 expression in oral cancer cells. Further studies of the mechanisms of action of 7ND are necessary, since osteoclast formation is inhibited, but it is unclear which kind of cells the affected monocytes differentiated into. The transcriptional factors of osteoclast formation, such as NFκB, AP-1 or NFAT may have also been inhibited.\textsuperscript{215,216} Another important need is for improved animal models of bone invasion by epithelial cancers. SCC25 is an aggressive cell line with high invasive properties, but a better validated animal model is necessary to reduce variance and improve the efficiency of animal experimentation in future.
In summary, the current studies attempted to investigate the signalling pathways involved in the progression of bone invasion by OSCC, but under limited conditions. There are, inevitably, a number of limitations. Local bone invasion and the formation of bone metastases are closely related pathological processes, so studies of the latter may inform the former. Nevertheless, invasion of bone in cases of oral cancer has its own special characteristics, and the behaviour depending, inter alia, on the site of origin, and local characteristic of the bone and the dentition. Our future research plan is to employ microarray analysis, to analyse all relevant genes associated with osteoclast recruitment, mobilization and function in cell lines derived from primary neoplasms with high potential for bone metastasis, such as those from breast, prostate, or lung cancer, and cell lines derived from oral cancers with or without bone invasion (Fig.6.1). Meanwhile, the internal comparison of gene files should be done between OSCC cell lines with or without bone invasion (Fig.6.1A), and the external comparison of gene files would also be done for OSCC cell lines of bone invasion and cell lines of bone metastasis (Fig.6.1B). Since not all the gene files are similar, some interesting genes would be chosen as candidates for future interactions to overcome this complication.
**Figure 6.1.** The blueprint of a future research plan to be performed by microarray analysis. (A) Internal comparisons of gene files will be made for oral cancer cell lines demonstrated to have high or low propensity for bone invasion; (B) External comparisons of gene files will be made with cell lines demonstrated to have the propensity to produce bone metastases; (C) Some common genes will be studied to explore the common signalling pathways identified.
Chapter 7

References


Appendix

Publications from PhD

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