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

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Summary

Aims: This study investigates whether matrix metalloproteinases (MMPs), specifically MMP-2 and MMP-9, interacting with other molecules important in osteoblast differentiation and osteoclastogenesis, could play important roles in the invasion of bone by oral squamous cell carcinoma (OSCC).

Methods: Supernatant (conditioned medium, CM) was collected from OSCC cell lines (SCC15 and SCC25), and from cultured osteoblasts (hFOB cell line and a primary culture, OB), and used for indirect co-culture: OSCC cells were treated with CM from osteoblasts and vice versa. Zymogenic activities of MMP-2 and -9, and protein quantities of all molecules studied, were detected by gelatine zymography and Western blotting, respectively. Real-time polymerase chain reaction (PCR) analysed mRNA of these molecules. Targeted molecules were examined by immunohistochemistry in tissue sections of bone-invasive OSCCs.

Results: Zymogenic activities of both MMPs were increased in OSCC cells following culture with CM from hFOB: Twist1 protein expression 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 lines, while zymogen expression of MMP-2 was decreased. Real-time PCR showed generally similar changes in expression of these molecules. All targeted molecules were expressed in invading malignant keratinocytes, and all but OPG were expressed in osteoclasts of clinical samples.

Conclusions: Crosstalk between different cell types appears to exist in the invasion of bone by OSCC. Understanding and ultimately interfering with the molecules involved may provide therapeutic approaches to inhibit such bone invasion.

Key words: Bone invasion, immunohistochemistry, MMPs, OPG, oral squamous cell carcinoma, osteoblasts, osteoclasts, RANKL, Runx2, Twist1.

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INTRODUCTION

Oral cancer is the sixth most common cancer worldwide, with a poor 5-year survival rate of only 50–60%. More than 90% of oral malignancies are squamous cell carcinomata (OSCC).[1,2] Lesions arising in the gingiva, retromolar trigone, buccal mucosa or floor of mouth may extend to bone of the maxilla and/or the mandible.[3,4] 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.[5,6]

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.[7–9] Generation of osteoclasts in the presence of tumour cells is likely to be due to an indirect effect involving stimulation of osteoblasts.[7–9] Osteoblasts produce a family of tumour necrosis factor-like (TNF) proteins and receptor molecules (TNFR): 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.[10] 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.[11]

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.[12–14] Matrix metalloproteinases (MMPs) are the main proteases responsible for digestion of fibrillar and non-fibrillar collagens, elastin, gelatine and proteoglycans.[15,16] 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.[17]

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.[18,19] 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 of incubation,

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which might facilitate survival of these cells within bone.[20] Twist1 is a negative transcription factor which inhibits osteoblast differentiation, maintaining these cells in the osteoprogenitor state.[21] Twist1 would competitively interact with Runx2, which acts as a master regulator, essential in the initial differentiation of mesenchymal cells into osteoblasts.[22] 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 overexpression of Twist1 has been shown to increase the invasion of gastric carcinoma cells by promoting MMP-2 secretion,[23] and high levels of Runx2 have been shown to increase expressions of MMP-9 and MMP-13 in prostate cancer cells.[24] 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 hypothesised 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.

MATERIALS AND METHODS

Materials

DMEM/F12 medium, fetal bovine serum (FBS), trypsin-EDTA and the primary antibody of anti-alkaline phosphatase (ALP) were purchased from Invitrogen (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).

Cell lines and culture conditions

The OSCC cell lines SCC15 and SCC25 were kind gifts of Associate Professor Nick Saunders (Princess Alexandra Hospital, Brisbane, Australia). The immortalised human fetal osteoblast cell line hFOB was obtained from American Type Tissue Collection (ATCC; Rockville, USA). OSCC cells were maintained in DMEM/F12 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% CO_2/20% O_2). hFOB cells were grown in DMEM/F12 with 10% FBS plus 300 μg/mL gentamicin at 34°C.

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 the Human Research Ethics Committee of Griffith University. 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 until cells became confluent. OB cells were characterised by immunocytochemical detection of ALP and by Von Kossa staining.

Indirect co-culture

SCC15, SCC25, hFOB or OB cells were firstly seeded individually at a density of 1.5×10^6 cells in 75 cm^2 flasks and cultured overnight. The culture medium was then changed to DMEM/F12 without FBS. After a further 48 h culture, supernatant from each cell line was collected and centrifuged at 1500 g for 20 min at 4°C to remove cell debris, and used as conditioned medium (CM)[25] in the following indirect cell co-culture. All cells were plated individually at a density of 2×10^5 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 48 h culture, CM, and mRNA and protein extracted from the residual cells of each cell line were collected for the experiments described below.

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 Laboratories, 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 $CaCl_2$; 0.02% Brij-35) at 37°C for 40 h. Finally, gels were stained with Coomassie Brilliant Blue R-250 (Sigma, USA) for 1 h at room temperature and progressively destained until clear bands appeared against the blue background.

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 (Pierce, USA). Briefly, 40 μg of protein was subjected to 10% SDS-PAGE precasting gel (Bio-Rad Laboratories). 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).

Real-time PCR

After the indirect co-culture, total RNA from OB or OSCC cells was extracted using the PureLing RNA Mini Kit (Invitrogen). The RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). 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 (Invitrogen) and the icycler iQ5 Real-time PCR system (Bio-Rad). The data were normalised to the internal control, GAPDH, to obtain ΔCt. The final amount of gene of interest relative to untreated samples was reported by the $2^{-\Delta\Delta Ct}$ method.[26] Primers used in this study are listed in Table 1.

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%

Table 1 Primer sequences used in real-time PCR

Genes	Primers	Length
RANKL	Forward: 5′-CAG AAG ATG GCA CTC ACT GCA-3′ Reverse: 5′-CAC CAT CGC TTT CTC TGC TCT-3′	203 bp
OPG	Forward: 5′-GGA ACC CCA GAG CGA AAT ACA-3′ Reverse: 5′-CCT GAA GAA TGC CTC CTC ACA-3′	225 bp
MMP-2	Forward: 5′-GACATACATCTTTGCTGGAGAC-3′ Reverse: 5′-TTCAGGTAATAGGCACCCTT-3′	180 bp
MMP-9	Forward: 5′-CTTCACTTTCCTGGGTAAG G-3′ Reverse: 5′-CACTTCTTGTCGCTGTCAAA-3′	105 bp
Twist1	Forward: 5′-TGT CCG CGT CCC ACT AGC-3′ Reverse: 5′-TGT CCA TTT TCT CCT TCT CT GGA-3′	63 bp
Runx2	Forward: 5′-CCA GAT GGG ACT GTG GTT ACT-3′ Reverse: 5′-ACG GTT ATG GTC AAG GTG AAAC-3′	164 bp
GAPDH	Forward: 5′-TGC ACC ACC AAC TGC TTA GC-3′ Reverse: 5′-GGC ATG GAC TGT GGT CAT GAG-3′	87 bp

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, Australia) for a few minutes at room temperature. Primary antibodies were replaced by non-immune serum as negative controls. Sections were counter-stained with Mayer's haematoxylin, dehydrated, and mounted with DPX (BDH Laboratory, England). The final results were visualised by light microscopy (Leitz Laborlux S; Leitz, Germany) and photographed using a Nikon OXM1200 digital camera with the Act-1 program (Nikon, Japan).

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.

RESULTS

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. 1). Preliminary studies using primary human dermal fibroblast cells as a negative control revealed no differences (data not shown).

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. 2).

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. 3A,B). After 20 days of continuous culture *in vitro*, Von Kossa staining revealed deposition of calcium salts, reflecting their osteogenic properties (Fig. 3C,D).

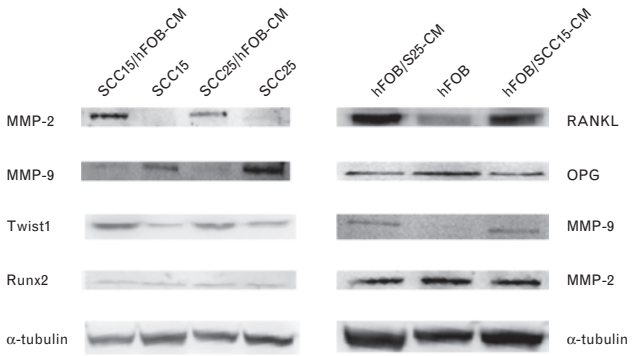

Fig. 2 Western blotting analysis of the indirect co-culture of OSCC and hFOB cells. MMP-2 protein level was increased, while MMP-9 was decreased in both OSCC cell lines. Twist1 protein expression was increased while no change to protein expression of Runx2 was found. In hFOB cells, RANKL protein expression 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.

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. 4).

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 into the bone, and osteoclasts had accumulated in resorption lacunae (Fig. 5A). Immunohistochemistry 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. 5B,C). For Twist1, weak cytoplasmic expression was found in OSCC cells, and stronger cytoplasmic expression in osteoclasts (Fig. 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. 5E,F). Control sections were negative (data not shown).

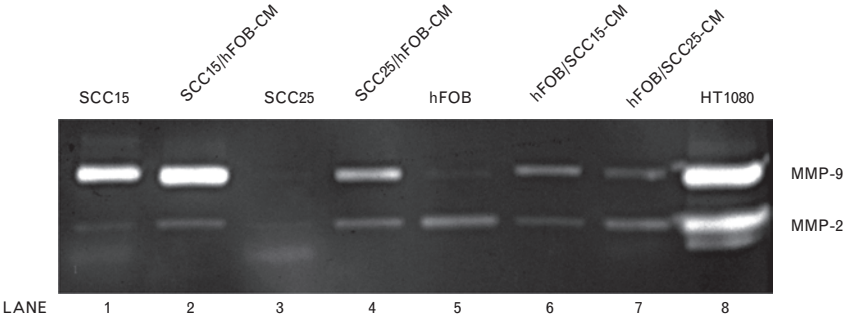

Fig. 1 Gelatine zymography results of the indirect co-culture of OSCC (SCC15 and SCC25) and hFOB cultured cells. The zymogenic activities of MMP-2 and MMP-9 were increased in OSCC cells treated with CM of hFOB (lanes 2 and 4); MMP-9 was increased while MMP-2 was decreased in hFOB treated with CM of OSCC cells (lanes 6 and 7). HT1080 cells were used as a positive control. This figure shown is typical of three independent experiments.

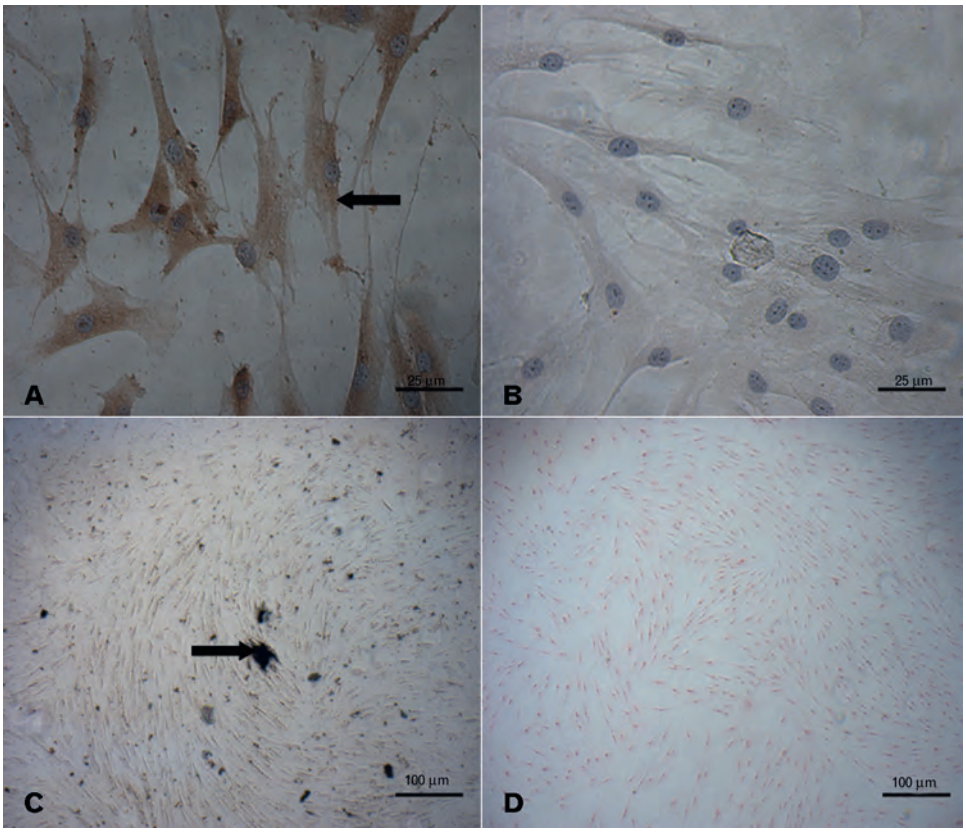

Fig. 3 Identification of the primary OB cells. (A) ALP staining was mostly localised in cytoplasm (arrow, DAB, bar = 25 μm). (B) No staining was found in the negative control (bar = 25 μm). (C) Various dark calcium deposits appeared in the culture plate after 20 days of continuous culture (arrow, bar = 100 μm). (D) No calcium deposits were found in the negative control (bar = 100 μm).

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.[27] Ishikuro *et al.* found, also in co-culture experiments, that BHY cells stimulated expression of RANKL mRNA in mouse osteoblasts.[28] 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.[29] 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.[30] 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*.[31]

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 SCC15 and SCC25 cells. Regulation of MMP-2 and MMP-9 is a complex process, operating at the levels of gene expression, post-translational stabilisation and zymogen activation.[32] 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.[33] 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.[34] Furthermore, the BHY cell line, which has bone invasive properties, has been shown to have high levels of Twist1 expression.[35] It has been reported that Twist1 would suppress Runx2 to inhibit osteoblast differentiation at an early stage of bone formation.[36] 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.[37] The reasons for the lack of response of Runx2 in our study are unclear; other

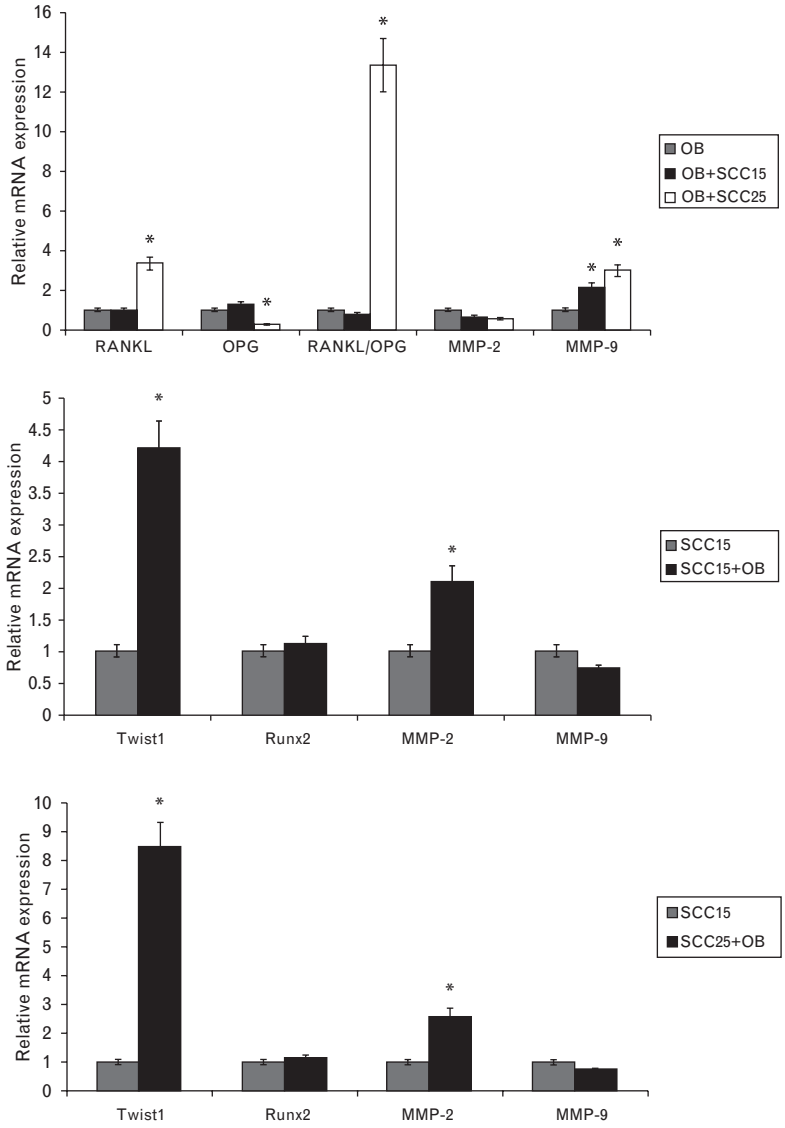

Fig. 4 Real-time PCR results of the indirect co-culture of OSCC and OB cells. The RANKL/OPG ratio was significantly increased in OB treated with CM of SCC25 ($p < 0.05$), while CM of SCC15 had no similar effect ($p > 0.05$). The expression of MMP-2 was decreased without significance ($p > 0.05$), and 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 SCC15 and SCC25. Data were shown as mean ± standard deviation of three independent experiments (*$p < 0.05$).

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.[10,11] 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.[38] 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,[39,40] the changes we have observed in these molecules suggest differentiation of osteoclasts and thus bone resorption.[41] This could be a component of the uncoupling of bone formation and bone resorption which has been shown to occur in cancer progression.[42]

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 utilised the indirect co-culture model to investigate if culture supernatant from OSCC cells up-regulated 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

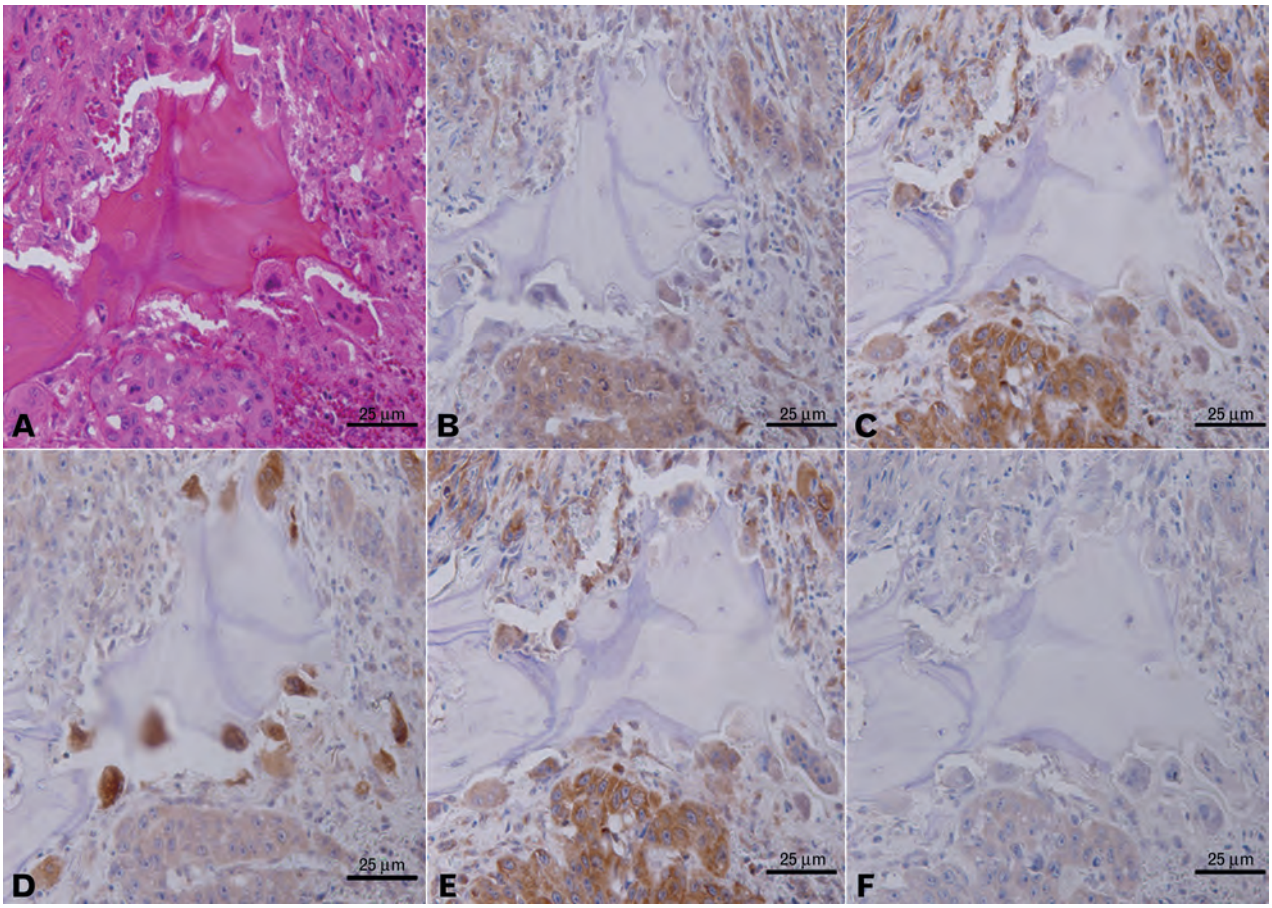

Fig. 5 Immunohistochemical analysis of targeted molecules in sections from OSCC with bone invasion. (A) H&E staining demonstrated an infiltrative pattern of bone invasion by OSCC. (B) MMP-2 was weakly expressed in the cytoplasm of OSCC cells (DAB, bar = 25 μm). (C) MMP-9 was strongly expressed in the cytoplasm of OSCC cells and weakly in the cytoplasm of osteoclasts (DAB, bar = 25 μm). (D) Weak Twist1 expression was found in cytoplasm of OSCC cells, with stronger expression in the cytoplasm of osteoclasts (DAB, bar = 25 μm). (E) The expression of RANKL was similar to that of MMP-9 (DAB, bar = 25 μm). (F) Weak expression of OPG was detected in the cytoplasm of OSCC cells and no expression was seen in osteoclasts (DAB, bar = 25 μm).

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.[43] The expression of focal adhesion kinase (FAK) is also found in SCC25, which may indicate a more invasive potential.[44] However, whether these characteristics of these particular cells affect expressions of the molecules we targeted here are 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 localised 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 that 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 resorptive properties. Clinical samples showed that nearly all of 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.

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