

# Epithelial-mesenchymal transition in oral squamous cell carcinoma triggered by transforming growth factor- $\beta$ 1 is Snail family-dependent and correlates with matrix metalloproteinase-2 and -9 expressions

BIN QIAO, NEWELL W. JOHNSON and JIN GAO

Griffith Institute for Health and Medical Research, and School of Dentistry and Oral Health, Gold Coast Campus, Griffith University, QLD 4222, Australia

Received April 12, 2010; Accepted May 25, 2010

DOI: 10.3892/ijo\_00000715

**Abstract.** Snail and Slug play important roles in cancer progression by promoting epithelial-mesenchymal transition (EMT). Although Snail is well studied, its role in oral squamous cell carcinoma (OSCC) and its regulatory relationship with Slug remain unclear. We aimed to determine whether Snail and Slug interplay occurs in transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)-initiated EMT of OSCC cells, and to assess the expression of matrix metalloproteinases (MMPs) in this process. Three OSCC cell lines, SCC9, SCC15 and SCC25 were treated with recombinant TGF- $\beta$ 1 for 0-6 days. Activities of the EMT regulators, Snail and Slug, and of extracellular hallmarks, MMP2 and 9, were detected using real-time PCR and Western blots. An *in vitro* wound healing assay then assessed short-term EMT, whilst phenotypic changes associated with the above gene expressions were evaluated by immunocytochemistry. Results showed that Slug and MMP9 were up-regulated at both mRNA and protein levels, while Snail expression rose and fell, in concert with expression of MMP2. In the wound healing assay, Snail was seen alone at the invasive front in SCC9 and SCC15 cultures, while both Snail and Slug appeared at wound margins in SCC25. Both MMP2 and MMP9 were detected at wound edges in all cell lines, with MMP2 localised in cell nuclei, MMP9 was restricted to the cytoplasm. The study suggests that both Snail and Slug act as regulators of TGF- $\beta$ 1-triggered EMT in OSCC cells. Snail may up-regulate MMP2 or MMP9 initiating EMT, while Slug may share a role with Snail in maintaining longer-term EMT, by stimulating MMP9 expression.

## Introduction

Oral squamous cell carcinoma (OSCC) remains a major public health problem world-wide, with ~275,000 cases annually and little improvement in survival rates (1). Despite advances in treatment, facial disfigurement, and functional disturbances including mastication, swallowing, and speech remain distressingly common (2). It is critical to understand key molecular mechanisms in the transformation and spread of OSCC, with a view to designing targeted- or individualised-therapies (3,4).

Epithelial-mesenchymal transition (EMT) is attracting interest as part of the process of invasion and metastasis. EMT is recognised by changes in cell-shape, in which epithelial cells *in vivo*, become detached from each other, penetrate the basement membrane and acquire a more flexible and migratory phenotype akin to mesenchymal cells (5).

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) has been implicated in a contradictory array of biological processes, including both inhibition of cell proliferation and carcinogenesis. Many components of the TGF- $\beta$ 1 cascade function as tumour suppressors - activities that cancers must bypass by selective loss or mutation of the relevant gene (6). However, TGF- $\beta$ 1 is over-expressed in OSCC, and has been regarded as a key initiator of EMT (7). TGF- $\beta$ 1 acts not only through traditional Smads pathways (8), but also signals through non-Smad pathways, including the matrix metalloproteinase (MMP) cascade (9), to regulate gene expressions associated with malignant transformation.

MMPs, a large family of more than 28 enzymes, digest extracellular matrix (ECM) substrates and promote invasion of blood vessels and lymphatics in the spread of oral cancer (10). OSCC cells frequently express MMPs, and appear spindle-shaped at the invasive front of the neoplasm, with loss of E-cadherin expression, cardinal signs of EMT (11). Potential relationships between EMT and MMPs, particularly MMP3 and MMP9, have been extensively studied (12,13).

Snail family (Snail and Slug) factors play an important role in cancer progression by promoting EMT. Although Snail itself is well studied in human cancers, its role in mediating EMT in OSCC and its relationship with Slug remain unclear. Here, we investigate whether TGF- $\beta$ 1 can trigger the

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*Correspondence to:* Dr Jin Gao, Griffith Institute for Health and Medical Research, and School of Dentistry and Oral Health, Gold Coast Campus, Griffith University, QLD 4222, Australia  
E-mail: j.gao@griffith.edu.au

**Key words:** oral squamous cell carcinoma, epithelial-mesenchymal transition, transforming growth factor- $\beta$ 1, Snail family, matrix metalloproteinase-2, metalloproteinase-9

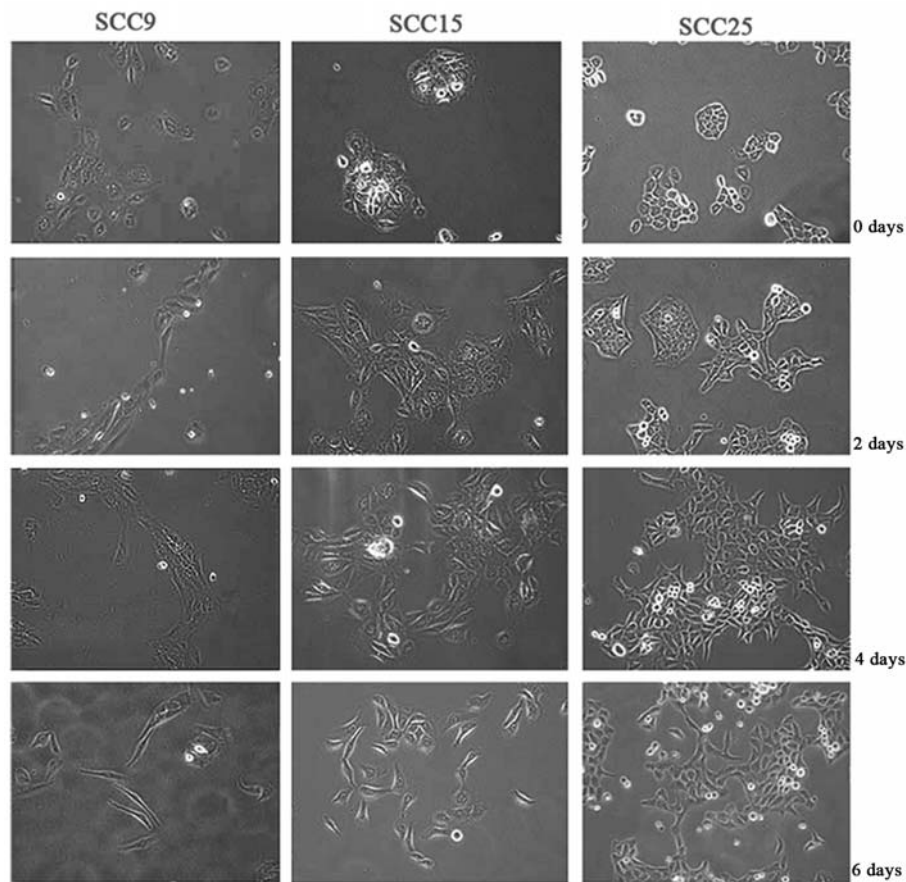


Figure 1. TGF- $\beta$ 1 activation induces morphologic changes consistent with EMT in SCC9, SCC15 and SCC25 cells. At day 2 following induction, obvious spindle-shaped cells appear in SCC9 and SCC15, however, the mesenchymal morphology is not obvious until day 6 in SCC25 (x200).

appearance of EMT in OSCC cells, and explore the potential of Snail family pathways in the process through variations in MMP2 and MMP9 expressions.

## Materials and methods

**Materials.** Recombinant TGF- $\beta$ 1 was from R&D Systems. DMEM/F12 medium, fetal bovine serum (FBS), and secondary antibodies for immunofluorescence including Alexa Fluor-532 goat anti-mouse IgG and Pacific Orange goat anti-mouse IgG were from Invitrogen. Primary monoclonal antibodies of mouse anti-E-cadherin and anti-Snail were from Santa Cruz Biotechnology; anti-Slug, anti-MMP2 and anti-MMP9 were from Cell Signalling Technology. Anti-vimentin and anti-N-cadherin were purchased from Boster (China);  $\alpha$ -tubulin antibody was from Abcam. Secondary antibodies for Western blotting, goat anti-mouse IgG and goat anti-rabbit IgG were from Bio-Rad.

**Cell culture.** Three OSCC cell lines, SCC9, SCC15 and SCC25 were the gift of Professor Nick Saunders, University of Queensland, and routinely maintained in DMEM/F12 containing 10% FBS. To examine the effects of TGF- $\beta$ 1 treatment, equal numbers of cells ( $1 \times 10^3$ /well) from the three lines were placed in serum-free medium, starved for 12 h, then induced with TGF- $\beta$ 1 at 5 ng/ml for 0, 2, 4, and 6 days. Culture medium was changed every other day.

**Immunofluorescence assay.** Cells were placed into 8-well slides, treated as above, fixed with absolute methanol for 10 min, and blocked in 5% bovine serum albumin for 30 min. Primary antibodies, vimentin (1:150) and E-cadherin (1:500) were incubated at 4°C overnight, followed by detection with fluorescence-conjugated secondary antibody at room temperature for 2 h. Images were acquired under a Nikon inverted microscope. Normal serum replaced primary antibodies as controls.

**Real-time PCR (qPCR).** Total RNA was isolated from cells using the PureLing RNA Mini Kit (Invitrogen, USA), the RNA reverse-transcribed to cDNA with iScript cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturer's instructions. Quantitative gene expression was performed for Snail, Slug, vimentin, E-cadherin, N-cadherin, MMP2 and MMP9 using TaqMan One-Step RT-PCR Master Mix Reagent Kit (Invitrogen) and the icycler iQ5 Real-time PCR system (Bio-Rad). Data were normalized to the internal control, GAPDH to obtain  $\Delta C_t$ . The final value of the gene of interest relative to the untreated cells was converted by the  $2^{-\Delta\Delta C_t}$  method. Primers used were as reported elsewhere (14-16).

**Western blot analyses.** Total protein was extracted from cells with lysis buffer (Bio-Rad). Proteins (40  $\mu$ g) were subjected to SDS-PAGE with 10% polyacrylamide gels and transferred to PVDF membranes. These were blocked with 5% non-fat

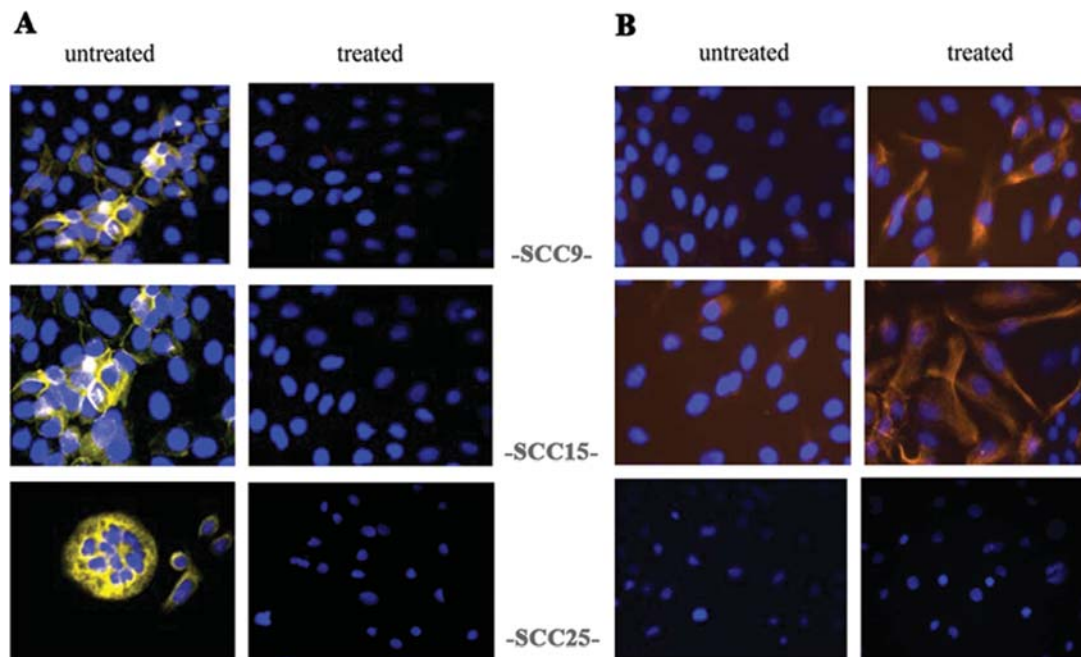


Figure 2. Immunofluorescence-staining shows localisation of E-cadherin to the cell membranes of all three cell lines. This is down-regulated sharply following treatment with TGF-β1 (A). Vimentin is expressed in the cytoplasm after treatment with TGF-β1 in SCC9 and SCC15 cells, but not in SCC25 cells (B) (x400).

milk for 2 h at room temperature, prior to incubation with primary antibodies, α-tubulin (1:3000), Slug (1:1000), Snail (1:200), E-cadherin (1:1000), N-cadherin (1:200), vimentin (1:200), MMP2 (1:500) and MMP9 (1:500) at 4°C overnight. Membranes were washed twice and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. Subsequently, protein bands were detected by enhanced chemiluminescence (ECL) and visualised using VersaDoc-MP Imaging Systems (Bio-Rad).

**Wound healing assay and immunocytochemistry.** Cells were seeded into 24-well plates and grown to confluence for the artificial wound healing experiment. A cell-free area was created by scratching with a pipette tip and the debris washed-out with PBS. Cultures were then treated with TGF-β1 (5 ng/ml) for 10 h, followed by fixation with absolute ethanol for 10 min and permeabilised with 0.1% Triton X-100 for 5 min. Cells were then incubated with primary antibodies, Slug (1:300), Snail (1:200), MMP2 (1:250) and MMP9 (1:250), respectively, for 12 h at 4°C and with secondary antibody at 37°C for 1 h. Colour was developed with diaminobenzidine (DAB) for 5 min and visualised by light microscopy.

**Statistical analysis.** Data from qPCR assays were analysed by One-way analysis of variance, using Statistical Analysis System 8.0. A  $P < 0.05$  was set as significant.

## Results

**TGF-β1 induces the characteristics of mesenchymal cells in OSCC cell lines.** During cultivation, all SCC cells gradually changed from slabstone to spindle-shaped and became scattered after 6 days of treatment (Fig. 1). Changes in cultures

of SCC9 and SCC15 appeared earlier, at day 2, than those in SCC25, which did not appear until day 6. Immunofluorescence showed that the expression of E-cadherin was decreased (Fig. 2A), while vimentin increased (Fig. 2B): SCC25 cells, however, differed: E-cadherin dramatically decreased whilst vimentin was not detected at all.

*The Snail family may be pivotal regulators of TGF-β1-triggered MMP2/9 expression.* Activities of the EMT-related molecules, Slug, N-cadherin and vimentin were all increased at mRNA and protein levels, whereas E-cadherin was down-regulated, in all three cell lines after treatment with TGF-β1. Trends with Snail differed (Fig. 3): expression was consistently elevated in SCC25 at protein level (Fig. 4C). In contrast, Snail expression appeared labile in SCC9 and SCC15 as it declined sharply after 2-day treatment (Fig. 4A and B). Although MMP9 was highly expressed, MMP2 varied markedly at mRNA and protein levels (Figs. 3 and 4). Interestingly, SCC25 cells reacted differently: expression of MMP2 was progressively elevated over time (Fig. 4C).

*Snail and Slug may share their individual roles in regulation of MMP2 or MMP9 in the process of EMT.* In this study, the relationships between Snail family activities and MMP production were assessed by the wound healing assay. Following stimulation with TGF-β1 for 10 h, cells migrated into the wound space. Snail proteins were restricted to cytoplasm in SCC9 cells at the migrating margin, to the nucleus in SCC15 and, in SCC25, again to cytoplasm. Slug proteins were seen only in SCC25, with nuclear location (Fig. 5A). MMP2 appeared to be nuclear in SCC9 and SCC15, but predominantly cytoplasmic in SCC25. MMP9 was perinuclear/cytoplasmic in all cell lines (Fig. 5B).



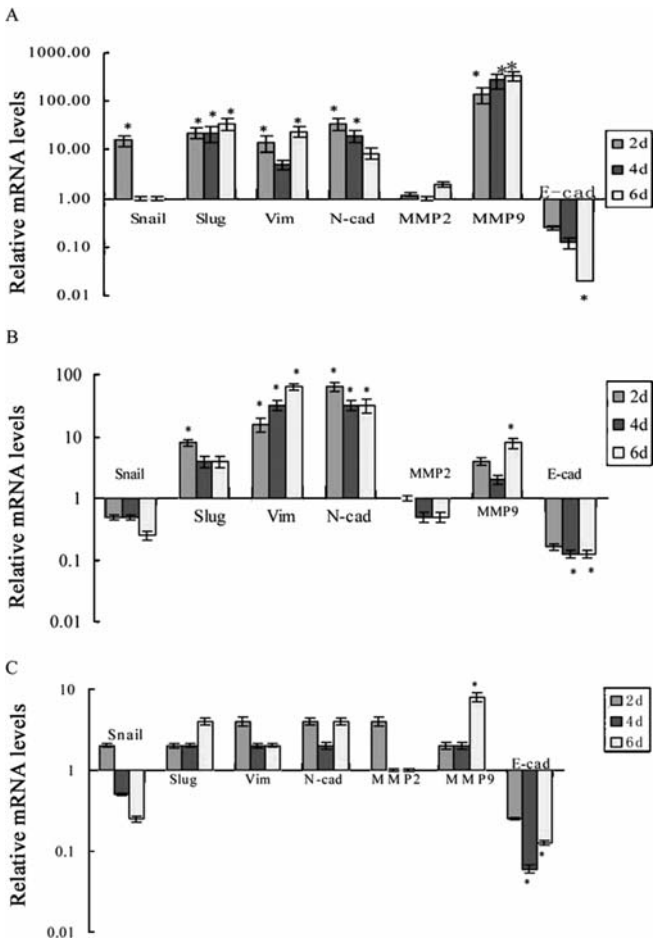


Figure 3. Relative mRNA levels, by qPCR assay, of Snail and Slug, and of the EMT markers vimentin and the switch from E- to N-cadherin following treatment with TGF- $\beta$ 1 for 2, 4 and 6 days. Levels for MMP2 and MMP9 are also shown. (A) SCC9; (B) SCC15; (C) SCC25. \*Significantly different from untreated sample at P<0.05.

Discussion

TGF- $\beta$  superfamily members and MMPs have long been regarded as important factors in cancer cell invasion and metastasis. TGF- $\beta$  can enhance the activities of MMPs, which then hydrolyse basement membranes through a process involving EMT (17). TGF- $\beta$ 1 and MMP2, or MMP9 have been widely accepted as important mediators, or initiators in many malignancies, including OSCC (18,19). Recent studies have indicated that EMT is part of the process of cancer cell detachment, migration and metastasis, in which several transcription factors including Snail/Slug, Twist, ZEB family (ZEB1, ZEB2) are involved (20). It has been suggested that the Snail family down-regulates E-cadherin, changes cytoskeleton directly and constructs a highly complex signal network in TGF- $\beta$ 1-triggered EMT (21). Besides the disassembly of cell-cell junctions and loss of epithelial polarity, the remodelling of cell-matrix adhesions, again mediated by MMPs, has been acknowledged as an important step for cancer cells to metastasise. Thus, this study was designed to test our hypothesis that EMT in oral cancer, initiated by TGF- $\beta$ 1, may be mediated by Snail and Slug, which in turn regulate MMP2 and MMP9 expression.

TGF- $\beta$ 1-induced expression of Snail and Slug has been reported variable in oral cancer cells (22). Our study showed increased expression of Snail in SCC25 cells at both mRNA (particularly day 2) and protein levels following TGF- $\beta$ 1 treatment, in contrast to other reports in this cell line (22). We found maximal expression of Snail at day 0 in SCC9 and SCC15, which declined dramatically thereafter. Little information is available in the literature on the unstable features of Slug. However, TGF- $\beta$ 1 has been reported to increase Slug expression in OKF4, OKF6, SCC25 and UMSSC1 cell lines (22), and this is consistent with our findings with SCC9

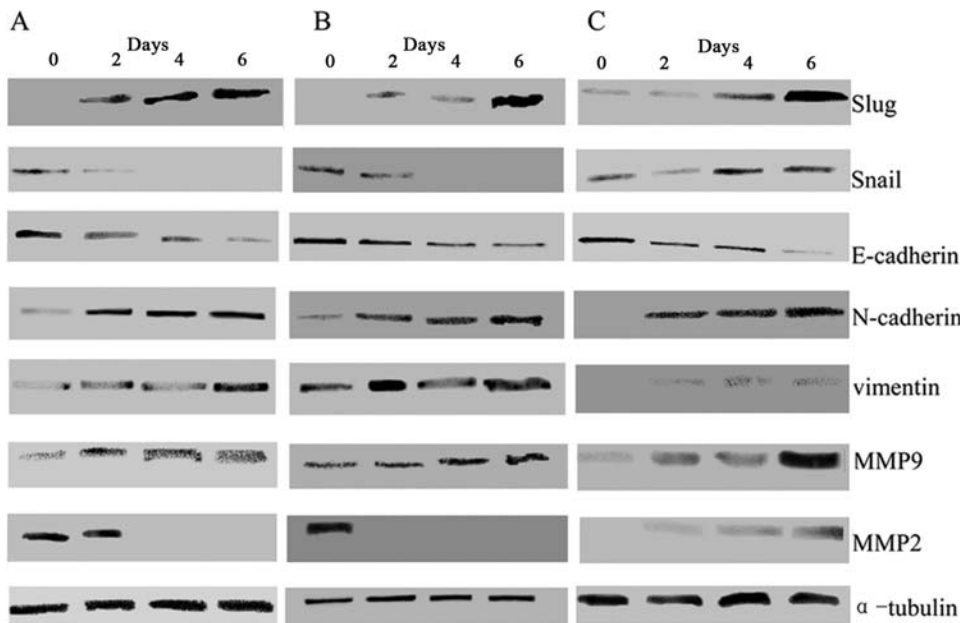


Figure 4. Western blots showing protein levels of all the markers under study at 0, 2, 4 and 6 days of stimulation with TGF- $\beta$ 1. (A) SCC9; (B) SCC15; (C) SCC25. Slug proteins increase with time in all cell lines, whereas Snail products fall in SCC9 and SCC15, but rise over time in SCC25. The switch from E-Cadherin to N-cadherin is clear. Vimentin increases in SCC9 and 15, but not obviously in SCC25. MMP9 production increases with time in all 3 cell lines. However, MMP2 products are disrupted in SCC9/15, but rise substantially in SCC25.

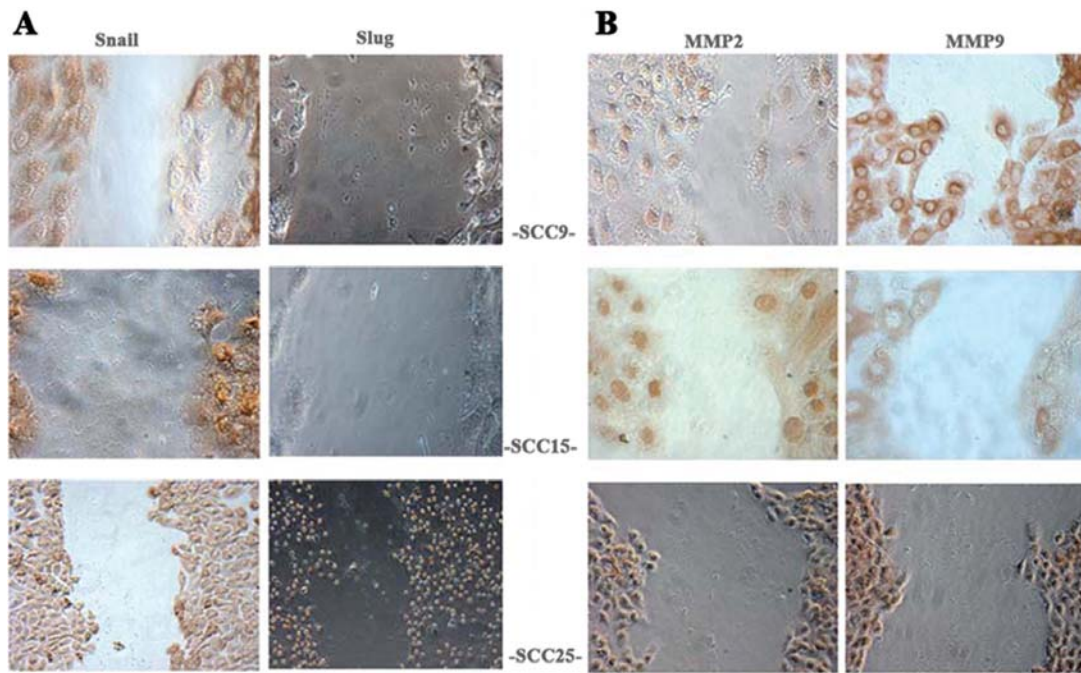


Figure 5. Photomicrographs of cell cultures 10 h after wounding, immuno-stained for Snail family proteins and for MMPs. Snail proteins are restricted to cytoplasm in SCC9 cells at the migrating margin, to the nucleus in SCC15 and, in SCC25, again to cytoplasm. Slug proteins are only seen in SCC25, with nuclear location. MMP2 appears to be nuclear in SCC9 and 15, but predominantly cytoplasmic in SCC25. MMP9 is perinuclear/cytoplasmic in all cell lines (x400).

and SCC15 lines. The reason why specific Snail expression is so variable in different OSCC cell lines is unclear (22,23). It is a difficult protein to detect, and most studies have relied on measurement of mRNA levels (23).

In addition to its variable expression over time, the sub-cellular distribution of Snail also varies. Theoretically, Snail should be seen in cell nuclei as a functional transcription factor, however it has been described in both cytoplasm and nucleus: these studies also describe five subtypes; wild-type Snail (Snail-WT) and variant Snail proteins (Snail-2SA, Snail-4SA, Snail-6SA, Snail-8SA) (23,24). Snail-WT and Snail-2SA were found in both the cytoplasm and the nucleus, whilst other variants were found in the nucleus only. Interestingly, the protein product of Snail-WT is readily degraded, whereas those derived from all other Snail variants are resistant to degradation, possibly because they are readily dephosphorylated (25). In the present study, Western blotting indicated that Snail was continuously expressed in SCC25 but degraded in SCC9 and SCC15. Meanwhile, broad cytoplasmic distribution of Snail in SCC9 and SCC25, and limited cytoplasmic expression in SCC15, was shown in our wound healing assay. Taken together, these findings suggest that Snail-2SA and Snail-WT are Snail subtypes, whose expression is specific to SCC25 and SCC9/SCC15, respectively.

Despite the fact that the loss of E-cadherin and the acquisition of N-cadherin, the so-called 'cadherin switch', is regarded as a hallmark of EMT (26), its upstream mediators are still ambiguous. Slug-expressing UMSSC1 cells have been reported to show preservation of E-cadherin levels (22), in contrast to the effect of Snail in these cells, in which E-cadherin expression was strongly repressed (27). In our experiments, the switch was consecutively from E-cadherin to N-cadherin,

although Snail disappeared sharply in SCC9 and SCC15. We therefore propose that Snail mainly controls the rapid-response, whilst Slug acts on maintaining long-term EMT.

Although MMP2 and MMP9 have been widely accepted as accelerators of cancer metastasis in OSCC, the molecular mechanisms underlying their up-regulation by the Snail family remain unclear (28). It seems that MMP9 can be regulated by both Snail and Slug (22,27), but there are few reports of their effects on MMP2. It is well known that MMP2 and MMP9 have similar molecular structures except that the precursor form of MMP2, termed pro-MMP2, requires cleavage of the N-terminal pro-domain to become active (29). This is a prerequisite for Snail-regulation of MMP2, and can be brought about by a number of mechanisms, for example nuclear factor kappa-B (NF- $\kappa$ B) (30). Interestingly, stabilization of Snail by NF- $\kappa$ B is a recent discovery (31). Hence, an indirect relationship between Snail and MMP2 via NF- $\kappa$ B, distinct from the direct transcriptional regulation seen for MMP9, is possible. In our study, qPCR and Western blot results showed that only MMP2 responded well to Snail fluctuations. The fact that MMP2 declined in SCC9/SCC15, but increased in SCC25 could be due to silent NF- $\kappa$ B in SCC9/SCC15 but activation in SCC25. Exploring this hypothesis is the subject of further work.

Whatever the long-term mechanisms, our wound healing assay, regarded as short-term EMT, illustrated that both MMP2 and MMP9 were detectable at wound edges in all cell lines; MMP2 was expressed in cell nuclei, MMP9 was restricted to the cytoplasm. MMP2 and MMP9 are normally expressed in cytoplasm: the nuclear presence of MMPs having long been overlooked, although MMP2 has been reported in nuclei (32). Importantly, membrane type 1-matrix

metalloproteinase (MT1-MMP), the major activator of pro-MMP2, has been found to co-exist with MMP2 in the same nuclei of hepatoma cells (33). Our study suggests that nuclear MMP2 could be the active form following stimulation by TGF- $\beta$ 1. Thus, it could be generalised that MMP2 and MMP9 were both activated quickly by Snail specifically, Slug expression being negative at the initial stage of EMT, as illustrated by immuno-cytochemistry. This could contribute to early local invasion of connective tissues, the first step of tumour metastasis. Thereafter, Slug appears to control MMP9 expression, resulting in continued breakdown of ECM and increased cell motility.

Taken together, both Snail and Slug are regulators of EMT initiated by TGF- $\beta$ 1 in OSCC cell lines, with complementary rather than redundant roles in tumour progression. It is also notable that Snail may control the initial stage of EMT by regulating both a 'cadherin switch' and expression of MMPs, while Slug shares roles with other members of its gene family in maintaining longer-term EMT.

## Acknowledgements

This work was supported by funds from Griffith University and China Scholarship Council (2008638009).

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