

Snail overexpression induces an epithelial to mesenchymal transition and cancer stem cell-like properties in SCC9 cells

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Local invasiveness and distant metastasis are critical factors that contribute to oral squamous cell carcinoma-related deaths. Increasing evidence has shown that the epithelial to mesenchymal transition (EMT) is involved in cancer progression and is associated with the 'stemness' of cancer cells. Snail is a transcriptional factor that can induce EMT and preserve stem-cell function, which may induce resistance to radio- and chemotherapies in the cells. In the present study, SCC9 cells were transfected with an empty vector or a vector encoding human Snail (SCC9-S). Overexpression of Snail induced SCC9 cells to undergo EMT, in which the cells presented a fibroblast-like appearance, downregulated the epithelial markers E-cadherin and β -catenin, upregulated the mesenchymal marker vimentin, and associated with highly invasive and metastatic properties. Furthermore, the induction of EMT promoted cancer stem cell (CSC)-like characteristics in the SCC9-S cells, such as low proliferation, self-renewal, and CSC-like markers expression. These results indicate that overexpression of Snail induces EMT and promotes CSC-like traits in the SCC9 cells. Further understanding the role of Snail in cancer progression may reveal new targets for the prevention or therapy of oral cancers.

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Oral squamous cell carcinoma (OSCC) is the most frequent type of cancer in the oral cavity and is associated with high morbidity and poor prognosis.^{1,2} Despite progress in surgery, chemotherapy, and radiotherapy, the 5-year survival rate has remained at 50–55% over the past several decades.³ Local or regional recurrences and distant metastases have a critical role in this process, and the mechanism underlying their occurrence remains poorly understood.^{2,4}

During metastatic progression, tumour cells lose cell–cell adhesion, detach from the primary site, invade the basement membrane, survive and circulate in the blood vessels, leave the bloodstream, and finally colonise in a new host environment to form micrometastases.^{2,5–7} A growing body of research strongly suggests that the epithelial to mesenchymal transition (EMT), which occurs normally during embryonic development, tissue remodelling, and wound healing, is a

critical early event in tumour invasion and metastasis.^{8–10} It is characterised by downregulation of epithelial markers, such as E-cadherin, and upregulation of mesenchymal markers, such as vimentin. During the process of EMT, epithelial cells acquire mesenchymal cell properties and show reduced intercellular adhesion and increased invasion.¹¹

The transcriptional repressor Snail, which is a zinc finger protein, first described in *Drosophila melanogaster*, can bind to the E-boxes in the human E-cadherin promoter and suppress its transcription.^{12,13} Snail has previously been implicated in triggering EMT during embryonic development, fibrosis, and tumour progression.¹⁴ This process also occurs in the progression of carcinomas (including oral carcinoma cells), following the downregulation of E-cadherin expression or co-expression of NBS1.^{13,15–17} Snail also has been linked to cell survival or apoptosis, cell polarity, cancer cell invasion,

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recurrence, and the acquisition of cancer stem cell (CSC)-like characteristics.^{14,18,19}

Increasing evidence suggests that tumours develop and progress from a small subset of cells with the ability to self-renew, which enter the cell cycle infrequently. They constitute a population that is intrinsically resistant to classic treatments that are designed to kill cycling cells.²⁰ Such cells are called CSCs and have been identified in many cancers, including colon, breast, liver, brain, prostate, pancreatic, head and neck, lung, and hematopoietic malignancies.^{14,21}

A relationship between the EMT and CSCs has been observed, with the evidence suggesting that EMT cells acquire stem cell-like traits and that CSCs exhibit a mesenchymal-like appearance in immortalised nontumorigenic mammary epithelial cells and breast cancers.²² Furthermore, CSCs have been identified in head and neck SCC. It has been shown that head and neck SCC contains a subpopulation of cancer cells that have self-renewing ability, form new tumours, and possess the properties of CSCs.⁴ However, an association between the EMT and CSCs induced by Snail in the SCC9 cells has not been examined previously.

Taking this evidence together, we insight into the mechanisms that overexpression of Snail induces cells to undergo the EMT and gain stem cell-like characteristics in OSCC. Investigating the role of Snail at the molecular level in this process may identify novel treatment strategies against the most common malignancy of the oral cavity.

MATERIALS AND METHODS

Cell Culture, Plasmid Construction, and Transfection

The human SCC9, which is derived from carcinoma of the tongue, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells from this line were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 400 ng/ml hydrocortisone (Sigma-Aldrich, St Louis, MO, USA) and antibodies (Invitrogen). A full-length cDNA for human Snail based on the published sequence (NM_005985.2) was amplified by RT-PCR, with the incorporation of *Hind* III (TaKaRa, Otsu, Shiga, Japan) and *Bam* HI (TaKaRa) restriction sites in the primers. After agarose gel electrophoresis, the fragment was recovered by gel extraction (OMEGA, Norcross, GA, USA) and ligated to the pMD18-T vector (TaKaRa). The expected PCR product was confirmed by sequencing. The entire coding sequence was then excised from the pMD18-T vector by digestion with *Hind* III and *Bam* HI and subcloned into the mammalian expression vector pEGFP-N1 (kindly provided by Zi-Lu Wang, Number U55762). The final construct was verified by sequencing. Positive clones were selected by kanamycin and amplified in bacterial culture media. All the plasmids were purified using the Endo-free Plasmid Mini Kit II (OMEGA). For transfection, the SCC9 cells were plated in six-well plates (Corning, Lowell, MA, USA), cultured in a complete growth

medium to 80% confluency, and then cultured in the medium without FBS for 12–16 h. The transfection was performed using 2 µg (per dish) of either pEGFP-N1 or pEGFP-N1-Snail and Lipofectamine 2000 (Invitrogen) reagent, according to the manufacturer's protocol. At 48 h post-transfection, the media was supplemented with 400 µg/ml G418 (Invitrogen). Two weeks later, the clones were picked and grown in six-well plates until confluency. Stable SCC9-pEGFP-N1 (SCC9 cells transfected with an empty vector (SCC9-N)) and SCC9-pEGFP-N1-Snail (SCC9 cells transfected with a vector encoding human Snail (SCC9-S)) cell lines were obtained and subcultured.

Quantitative Real-Time PCR

The total RNA was extracted using the TRIzol reagent (Invitrogen), and the resulting mRNA was reverse transcribed into cDNA using the 5 × PrimeScript RT Master Mix (TaKaRa) at 37 °C for 15 min and 85 °C for 5 s, according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed using 2 × SYBR Premix Ex Taq (TaKaRa) with a 7300 ABI Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 31 s for 40 cycles. The relative mRNA levels were analysed by the 2(-ΔΔCt) method with *GAPDH* as an internal control.²³ *GAPDH* (5'-GAAGGTGAAGGTCGGAGTC-3', 5'-GAGATGGTGTATGGGATTTC-3'), E-cadherin (5'-TACACTGCCAGGAGCCAGA-3', 5'-TG GCACCAGTGTCCGGATTA-3'), Snail (5'-GACCACTATGC CGCGCTCTT-3', 5'-TCGCTGTAGTTAGGCTTCCGATT-3'), β-catenin (5'-GCTGAAGGTGCTATCTGTCTGCTC-3', 5'-TG AACAAGACGTTGACTTGGATCTG-3'), vimentin (5'-TGAGT ACCGGAGACAGGTGCAG-3', 5'-TAGCAGCTTCAACGGC AAAGTTC-3').

Western Blotting

The cells were grown to 80–90% confluency in 25 cm² cell culture flasks (Corning). The whole-cell lysates were collected and their concentrations determined using the Bradford assay. Protein (20 µg)²⁴ was separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The blots were then probed with antibodies, such as anti-β-actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-E-cadherin (1:400, Santa Cruz Biotechnology), anti-β-catenin (1:500, Santa Cruz Biotechnology), anti-vimentin (1:500, Santa Cruz Biotechnology), and anti-Snail (1:500, Abcam, Cambridge, MA, USA). The membranes were developed by Immun-Star WesternC Kit (Bio-Rad, Hercules, CA, USA) products and bands were detected by exposure to film (Kodak, Japan). The relative protein levels were quantified by dividing the other bands by the β-actin bands.

Immunofluorescence Microscopy

The SCC9-N and SCC9-S cells were grown on glass coverslips, fixed with 4% paraformaldehyde (PFA),²⁵ permeabilised in 1% Triton X-100 for 15 min, blocked with 1% bovine

serum albumin for 30 min, incubated with antibodies specific for E-cadherin (1:100, Santa Cruz Biotechnology) and vimentin (1:100, Santa Cruz Biotechnology) or Snail (1:100, Abcam), followed by the appropriate secondary antibodies (1:50), and then further incubated with DAPI (1:1000, Invitrogen) for 2 min. The preparations were visualised using a Zeiss LSM-710 laser-scanning confocal microscope.

Wound-Healing and Invasion Assays

The cells were cultured in six-well plates to 90% confluency and scraped with a 10- μ l pipette tip in the central area. After wounding, the detached and damaged cells were carefully removed with phosphate-buffered solution (PBS), and the culture was changed to a serum-free medium. The wound closure was observed microscopically and photographed at the indicated time points. An analysis assessing the invasion of cells was performed using Transwell filters with 6.5-mm diameters and 8- μ M pore sizes (Costar, Lowell, MA, USA). The filters were precoated for 30 min at 37 °C with 25 μ l extracellular matrix (Sigma-Aldrich) gel mixed with dimethyl sulfoxide (1:1). The trypsinised cells (4×10^5) were washed with PBS, resuspended in the serum-free medium, and placed in the upper chamber, and a medium containing 10% FBS was used as a chemoattractant in the lower chamber. After incubating for 24 h at 37 °C in 5% CO₂, the cells were fixed in 4% PFA and stained with crystal violet (Sigma-Aldrich) for 30 min. The nonmigratory cells on the upper chamber were removed with cotton swabs, and the migratory cells present on the lower surface were counted in 10 random fields and photographed by field at $\times 100$ magnification under an inverted microscope (Olympus).

Adhesion Assay

The cells were counted after trypsinisation, and a total of 4×10^5 cells were plated per well for 1 or 2 h, respectively. The unattached cells were carefully removed by washing twice with PBS, and the attached cells were trypsinised again and counted under an inverted microscope (Olympus).

Flow Cytometry

For the cell cycle analysis, the cultured SCC9-N and SCC9-S cells were detached with trypsin at 37 °C, washed twice with cold PBS, resuspended in 1 ml of 75% alcohol to ensure that the cells were single-fixed, and then stored at -20 °C for 24 h. The prepared cells were washed with PBS again, stained with 100 mg/ml of propidium iodide (BD Pharmingen, San Jose, CA, USA) at 4 °C for 30 min, and analysed by flow cytometry using the BD FACS Calibur (BD Biosciences) and CellQuest Pro software (BD Biosciences). Except for the cell-cycle markers, the expression of CSC-like markers was also evaluated by flow cytometry. The cells were collected, resuspended in PBS containing 3% FBS, and labelled with human-fluorochrome-conjugated anti-CD24-PE (10 μ l per test, Beckman Coulter, Los Angeles, CA, USA), anti-CD44-PE-Cy5 (0.625 μ l per test, eBioscience, San Diego, CA, USA),

or anti-CD133-PE (10 μ l per test, Miltenyi Biotech, Auburn, CA, USA) for 30 min in the dark. The corresponding mouse immunoglobulins conjugated to PE or PE-CY5 (BD Pharmingen) were used as isotype controls in each experiment.

Colony-Forming Assays

Single-cell suspensions (2000 cells per dish) were seeded on 100-mm dishes (Corning) and cultured for 14 days. The media was changed every 4 days. The colonies were washed twice with PBS, fixed in 4% PFA, and stained with crystal violet overnight. The colonies were observed and counted under an upright microscope (Olympus). Aggregates of >50 cells were defined as a colony according to the system described by Ma *et al.*²⁶

Statistical Analysis

The data were represented as mean \pm s.d. from at least three independent experiments,²⁷ and analysed by independent-samples *t*-test using the SPSS 13.0. software (SPSS). *P*-value less than 0.05 was considered significant.

RESULTS

Overexpression of Snail Correlates with EMT

Snail has been shown to induce EMT, during which epithelial cells lose their polarity and convert to a mesenchymal phenotype.¹⁶ In this study, we transfected SCC9 cells with either an empty vector (SCC9-N) or a vector encoding human Snail (SCC9-S). Six weeks later, the stable SCC9-N and SCC9-S cell lines were obtained. Upon observation by microscopy, the SCC9-S cells showed typical fibroblast-like and spindle-shaped appearances, whereas the SCC9-N cells showed robust cellular junctions with typical 'cobblestone-shaped' and epithelial-like appearances (Figure 1a). To determine if the pattern of gene expression correlated with EMT, real-time PCR was performed. The results showed that in the SCC9-S cells, 1.8-fold Snail overexpression decreased the E-cadherin message levels by 1.7-fold ($P < 0.01$) and downregulated β -catenin expression by 2-fold ($P < 0.01$). In addition, the mesenchymal marker vimentin showed a 1.9-fold ($P < 0.05$) increase in mRNA levels compared with the SCC9-N cells (Figure 1b). Then, western blot analysis was examined. The increased expression of Snail (2.91-fold) in the SCC9-S cells resulted in a decrease of the epithelial markers E-cadherin (1.96-fold) and β -catenin (4.35-fold), but an increase of vimentin (3.11-fold, Figures 1c and d), which was consistent with the real-time PCR results. In addition, the cellular-staining pattern of E-cadherin and vimentin were examined between the two cell lines. As shown by the immunofluorescence analysis, the SCC9-S cells presented a fibroblast-like appearance, with decreased E-cadherin expression but increased vimentin staining. By contrast, the SCC9-N cells possessed an epithelial-like morphology and stained positively for E-cadherin, but weakly for vimentin (Figure 2). These results demonstrated that increased Snail expression was associated with the EMT in SCC9 cells.

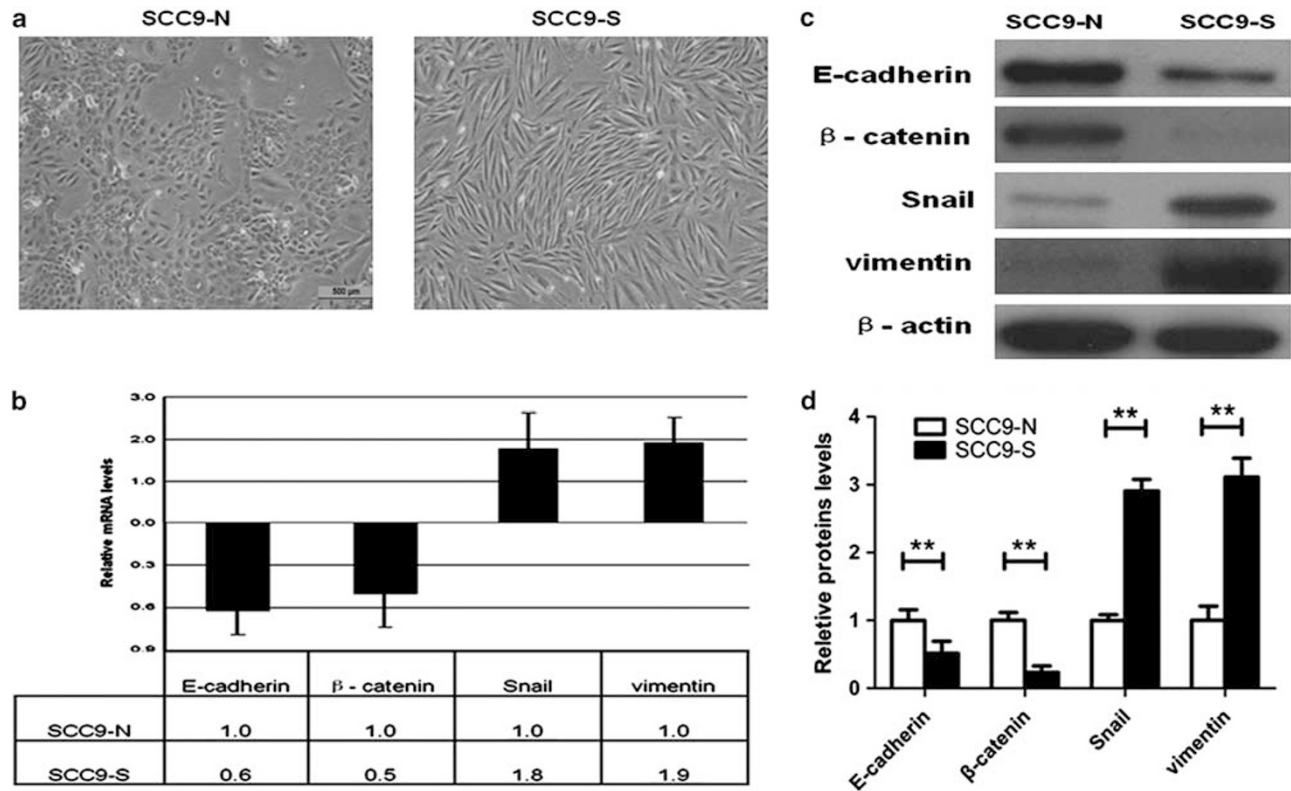


Figure 1 The overexpression of Snail significantly induces the epithelial to mesenchymal transition (EMT). (a) The SCC9 cells transfected with an empty vector (SCC9-N) presented typical cobblestone and epithelial-like appearances. The SCC9 cells transfected with Snail (SCC9-S) had a more elongated, fibroblast-like morphology compared with the controls. The photographs were taken at $\times 40$ magnification, and the scale bar = 500 μm . (b) A real-time RT-PCR was performed to determine the relative mRNA expression levels of E-cadherin, β -catenin, Snail, and vimentin, the housekeeping gene *GAPDH* was used as the control. The data were reported as mean \pm s.d. (c) The western blot analysis of the EMT-related proteins. E-cadherin, β -catenin, vimentin, and Snail were examined, β -actin was used as a loading control. (d) The relative proteins levels with E-cadherin, β -catenin, Snail, and vimentin. The data were reported as mean \pm s.d. ** $P < 0.01$.

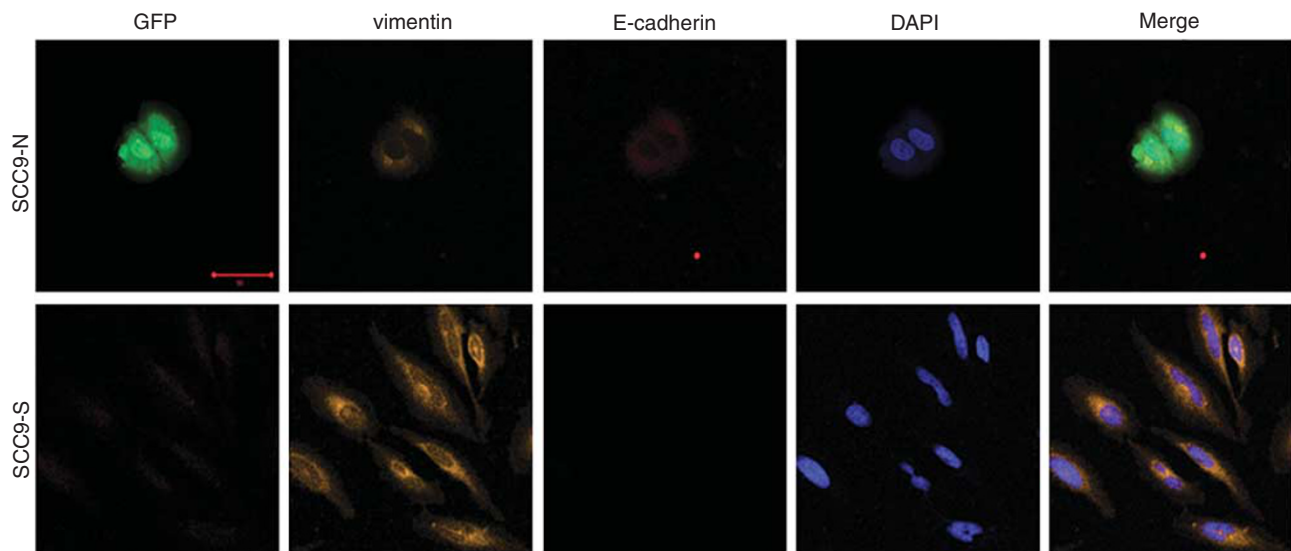


Figure 2 The double immunofluorescence staining of E-cadherin (red) and vimentin (yellow) in the SCC9 cells transfected with an empty vector (SCC9-N) and vector encoding human Snail (SCC9-S) cells. The end of the full-length cDNA for Snail was encoded with the terminator TGA, which blocked the expression of GFP (green) in the SCC9-S cells. The nuclei were stained with DAPI. The increasing expression of Snail in the SCC9-S cells induced a long, spindle-shaped morphology, and exhibited high vimentin expression, whereas the cells were negative for E-cadherin ($\times 200$ magnification). The scale bar = 50 μm .

The Cell Lines that Acquire an EMT Phenotype Show Highly Invasive and Metastatic Behaviour *In Vitro*

To test whether the mesenchymal cells were highly invasive and metastatic, Transwell assays were performed to measure single-cell invasion. After 24 h, the invasive cells were quantified and photographed at $\times 100$ magnification in 10 randomly chosen fields. The SCC9-S cells were more invasive than the SCC9-N cells ($P < 0.01$, Figure 3a) and showed a 4.9-fold increase in gel invasion (Figure 3b), which indicated that enhanced Snail expression contributes to this process. Additionally, the SCC9-S cells had lower adhesive ability than the SCC9-N cells (Figure 3c). The percentage of attached cells was 13.91% for the SCC9-S and 17.34% for the SCC9-N. One hour later, the percentage of attached cells increased in both cell lines to 16.09 and 29.06%, respectively. This

phenomenon was consistent with our observation that the SCC9-S cells were more easily trypsinised than the SCC9-N cells during subculturing. Furthermore, a wound-healing assay was used to assess the migration of the cells. The results showed that in contrast to the SCC9-N cells, the SCC9-S cells migrated less frequently and could not grow to confluency after 48 h (Figure 4).

Mesenchymal Cells Exhibit CSC-Like Properties

In this study, a cell-cycle analysis and colony-forming assay were performed to test the proliferative ability. As anticipated, the SCC9-S cells presented a significantly higher percentage of cells in the G0/G1 phases (64.75%) than did the SCC9-N cells (51.94%), but a lower percentage of cells in S phase (14.02%) than did the SCC9-N (32.91%, Figure 5a).

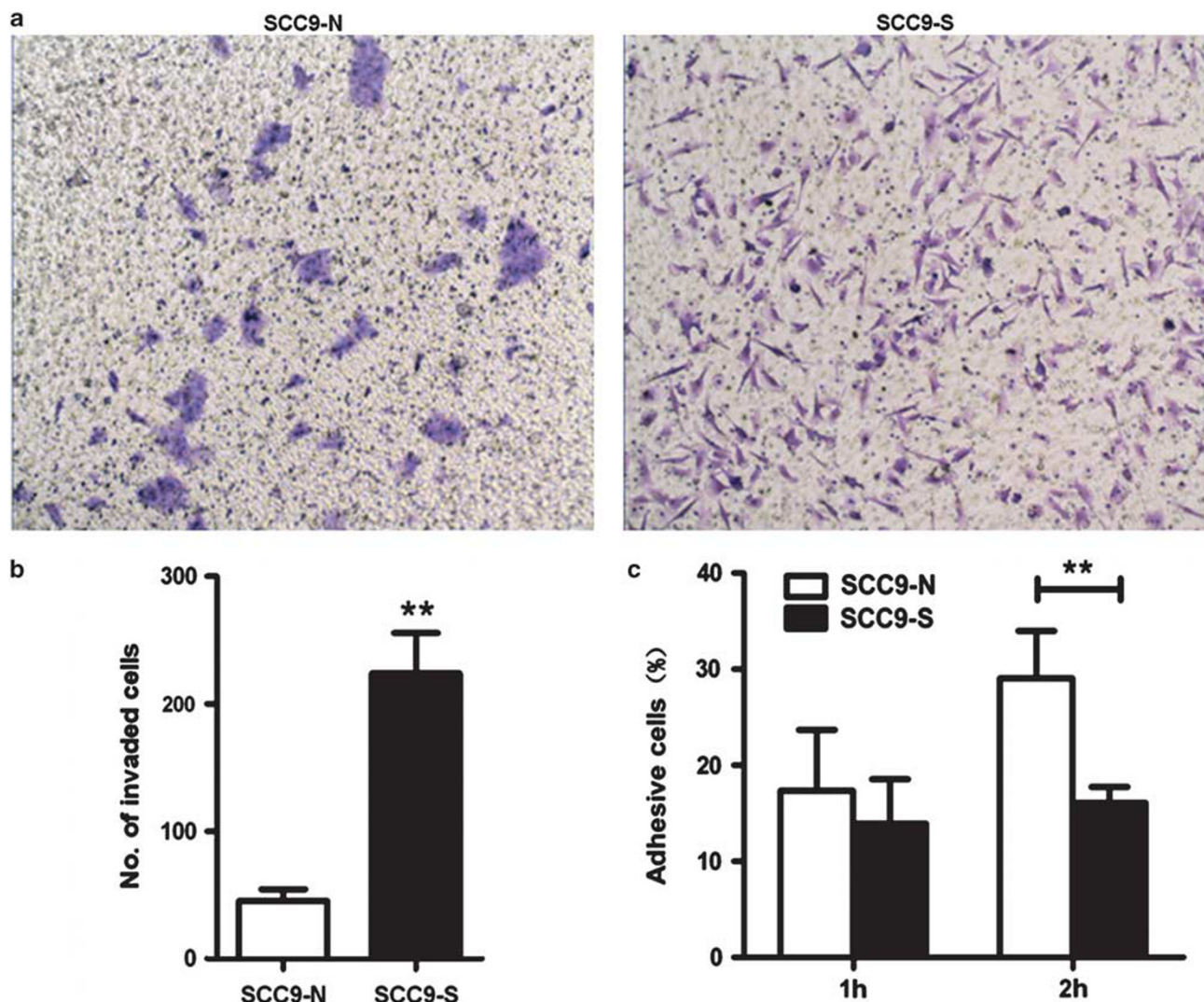


Figure 3 The SCC9-S cells that undergo the epithelial to mesenchymal transition (EMT) are associated with highly invasive and metastatic behaviour. (a) The cells invading the filters coated with the extracellular matrix (ECM) gel. The photographs were taken at $\times 100$ magnification. (b) The quantification of the cell invasion in 10 randomly chosen fields. The data were reported as mean \pm s.d. ** $P < 0.01$ compared with that of SCC9 cells transfected with an empty vector (SCC9-N) cells. (c) The quantification of cell adhesion at two time points. The data were reported as mean \pm s.d. ** $P < 0.01$.

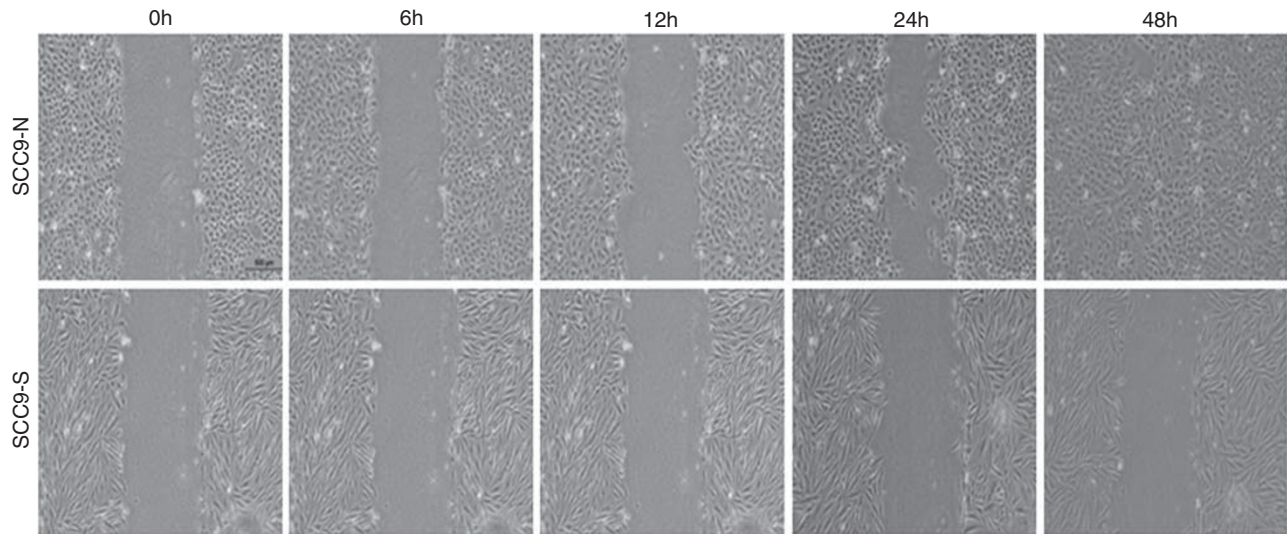


Figure 4 The capacity of the cells to fill the scratched area was examined under microscopy at the indicated time points. Confluent cell monolayers were scraped by a pipette tip in the central area. The SCC9 cells transfected with a vector encoding human Snail (SCC9-S) cells did not close the wound after 48 h. The photographs were taken at $\times 40$ magnification, and the scale bar = 500 μm .

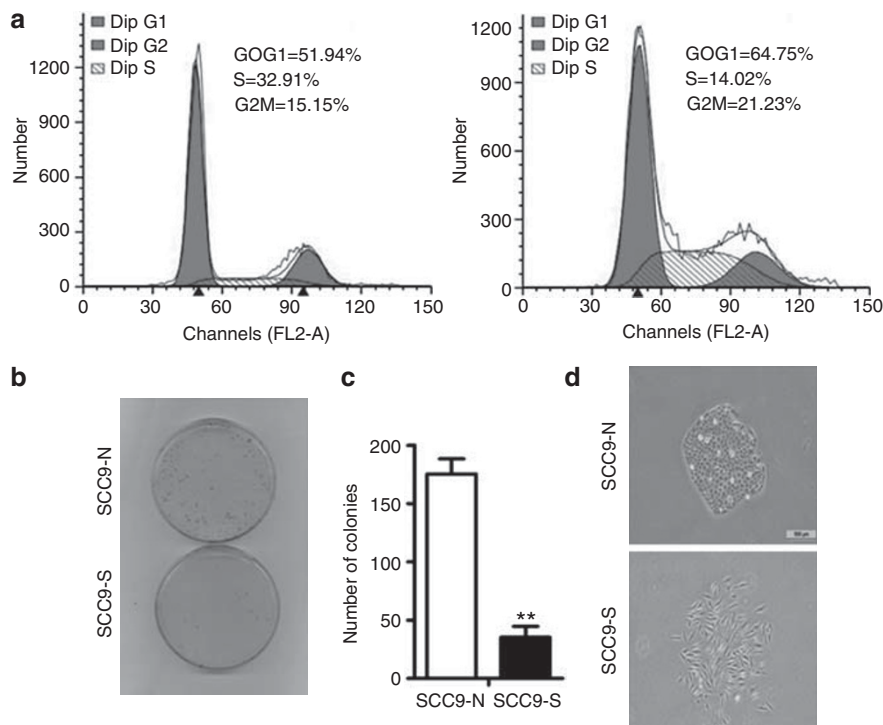


Figure 5 The SCC9 cells transfected with a vector encoding human Snail (SCC9-S) cells present low proliferation, but are self-renewable. (a) In the SCC9 cells transfected with an empty vector (SCC9-N) cells, 51.94% of the cells were arrested in the G0/G1-phase, in the SCC9-S cells, 64.75% of the cells were arrested in the G0/G1-phase, indicating that cell mitosis was blocked at the G0/G1 checkpoint and cell proliferation was inhibited. (b) Images of colonies stained with crystal violet. (c) The quantification of the colonies formed by the two cells, indicating that the SCC9-S cells had low proliferation ability. The data were reported as mean \pm s.d. $**P < 0.01$ compared with that of SCC9-N cells. (d) The SCC9-S cells can form colonies, indicating that they are self-renewable. The photographs were taken at $\times 40$ magnification, and the scale bar = 500 μm .

The colony-forming assay presented that the SCC9-S cells proliferated less frequently (Figure 5b), showed a 5.0-fold decrease in colony formation compared with the SCC9-N cells ($P < 0.01$, Figure 5c). In addition to using proliferation

ability, self-renew ability was also measured by the colony-forming assay. Although the SCC9-S cells displayed low proliferative ability, they showed self-renewal that can maintain the population of tumorigenic cells (Figure 5d). By

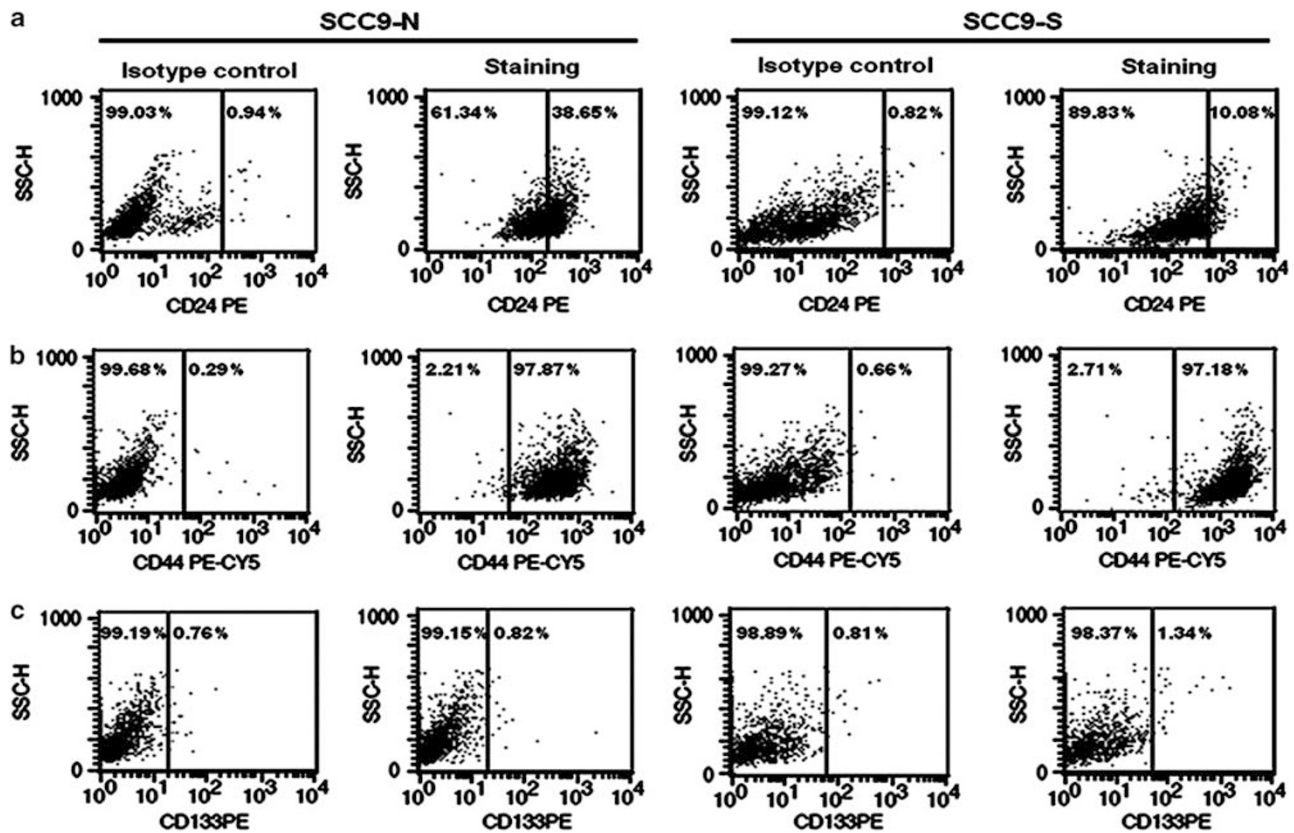


Figure 6 The overexpression of Snail generates a cancer stem cell (CSC)-like phenotype in SCC9 cells transfected with a vector encoding human Snail (SCC9-S) cells. The flow cytometry analysis of the CD24, CD44, and CD133 expression in the SCC9-S and SCC9 cells transfected with an empty vector (SCC9-N) cells. (a) The expression of CD24 was downregulated in the SCC9-S cells. (b) A high level of CD44 expression was assessed in the two cell lines. (c) CD133 expression was not detected in the two cell lines. Mouse immunoglobulins conjugated to PE or PE-CY5 were used as isotype controls in each experiment.

extension, we speculated that Snail-induced cells that undergo EMT may have stem cell-like properties. Then, CSCs surface markers expression for these cells was examined using flow cytometry. As shown in Figure 6, CD24 expression was lower in the SCC9-S cells (9.26%) than in the SCC9-N cells (37.71%, Figure 6a). However, there was no significant difference in CD44 expression between the two cell lines, the percentage of SCC9-S cells expressing CD44 was 96.52%, and in SCC9-N cells, it was 97.58% (Figure 6b), and in this study, SCC9-N and SCC9-S cells showed no significant CD133 expression (Figure 6c). Thus, the SCC9-S cells presented as CD133-negative and CD44^{high}/CD24^{low}, and the SCC9-N cells presented as CD133-negative and CD44^{high}/CD24⁺.

DISCUSSION

OSCC is a highly invasive and an aggressive cancer.¹ Recently, the EMT has been found to contribute to the progression and confer cancer cells with stem cell-like properties, in which the cells have a propensity for invasion, metastatic dissemination, resistance to certain therapy, persistence in tumours, and recurrence.^{19,28} Understanding the molecular and cellular changes may generate novel targets that can overcome the

aforementioned issues and reduce mortality. In this study, we made three novel observations that may help develop new treatments for OSCC: overexpression of Snail induced the SCC9 cells to undergo EMT; furthermore, these cells exhibited mesenchymal properties and were highly invasive, but not highly proliferative; and the induction of EMT conferred CSC-like properties to these mesenchymal cells.

In this study, the SCC9 cells that overexpressed Snail adopted mesenchymal cell properties. SCC9-S cells presented fibroblastoid phenotypes, had a more extended and spindle-like shape than did the SCC9-N cells. These significant morphological changes combined with the functional analysis demonstrated that the epithelial tumour cells underwent the EMT, in which the epithelial markers (such as E-cadherin and β -catenin) were downregulated and the mesenchymal markers (such as vimentin) were upregulated. Importantly, the SCC9-S cells acquired high invasive properties in our Transwell analysis. Thus, overexpression of Snail not only triggered the EMT in SCC9 cells, but also promoted its progression. Our findings in SCC9 cells are consistent with the prior studies have shown that Snail has a crucial role in EMT and is associated with an invasive ability in various human cancers.^{1,12,13,29–31}

EMT was also accompanied by the losing of cell adhesion and an increasing in cell motility.³² As shown in adhesion assays, overexpression of Snail inhibited cell–cell contacts. The SCC9-S cells exhibited a scattered phenotype and loss of adhesion, which contributes to cells becoming motile and correlates with their metastasis. However, the wound-healing assays revealed the novel results. The SCC9-S cells were highly invasive and metastatic, but they did not migrate into the scratched area after 48 h in culture due to their limited proliferation. This finding was consistent with the cell-cycle analysis, showing that the cell mitosis was blocked at the G0/G1 checkpoint and reduced in the S phase, which decreased the rate of cell proliferation in SCC9-S cells. In other words, Snail could be considered to be a master gene in the EMT, and led to invasive properties^{13,33} but low proliferation rates in the SCC9-S cells. Such novel results are supported by a previous study that shows Snail-expressing epithelial cells that have undergone EMT have a low proliferation potential.^{18,34,35} Our findings also present a likely explanation for the increased invasion, but decreased proliferation in the EMT: the SCC9-S cells are relatively quiescent, and composed of many cells in G0 phase that divide infrequently and may intrinsically resistant to current drug and radiotherapies designed to inhibit rapidly dividing cells.²⁰ On the basis of the current study, we speculate that overexpression of Snail in SCC9 cells induces the EMT and promotes CSC-like properties in mesenchymal cells, which correlates with the conclusion of Mani *et al*,²² and is also supported by Santisteban *et al*,³⁶ suggesting that the *in vitro* induction of EMT can generate cancer stem-cell properties from differentiated epithelial tumour cells.

Self-renewal, which is one of the hallmarks of CSCs, has been demonstrated in CSCs through their ability to form colonies.^{20,21} In this study, though the SCC9-S cells grew slowly in the serum-free culture media, they were able to form colonies, which is consistent with stem-cell properties. Moreover, the SCC9-S cells exhibited a CD24^{low} expression, but CD24⁺ expression in the SCC9-N cells, showed that 'stemness' is associated with CD24^{–/low} cells.³⁶ Prior studies showed that CD44 and CD133 had been used to isolate CSCs from multiple tumor types.^{21,37–39} However, in the present study, both of the cell lines presented as CD44^{high}, and showed only 0.53% of the SCC9-S cells expressed CD133, in contrast to 0.06% of the SCC9-N cells. With these findings in mind, a 'stemness' marker that works well in one cancer may not apply to other types of cancers.²⁰ It would be worthwhile to explore the values of CD44 and CD133 in combination with other cell surface markers for efficiently isolating CSCs^{21,40} in SCC9 cells.

In conclusion, SCC9 cells that overexpressed Snail undergo EMT, which presents mesenchymal phenotype and leads to invasive and metastatic properties. Furthermore, the mesenchymal cells show low proliferation rates, but still have self-renewal ability, and present decreased CD24 expression. These findings indicate that there is a link between the EMT

and CSCs in SCC9 cells. Importantly, Snail has a critical role in this process. Further exploring this mechanism may not only provide new insight into oral cancer research, but also identify a useful molecular target for efficiently treating this disease.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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