

RESEARCH PAPER



Periodontal pathogens promote epithelial-mesenchymal transition in oral squamous carcinoma cells *in vitro*

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ABSTRACT

Epithelial-mesenchymal transition (EMT) is potentially involved in increasing metastasis of oral squamous cell carcinoma (OSCC). Periodontal pathogens are well-known for their ability to induce intense immune responses and here we investigated whether they are involved in inducing EMT. Cultures of OSCC cell line (H400) were treated separately with heat-killed periodontal pathogens *F. nucleatum*, or *P. gingivalis* or *E. coli* LPS for 8 d. EMT-associated features were assayed using sq-PCR and PCR-arrays, for EMT-related markers, and ELISAs for TGF- β 1, TNF- α , and EGF. The migratory ability of cells was investigated using scratch and transwell migration assays. E-cadherin and vimentin expression was assessed using immunofluorescence while Snail activation was detected with immunocytochemistry. In addition, the integrity of the cultured epithelial layer was investigated using transepithelial electrical resistance (TEER). PCR data showed significant upregulation after 1, 5, and 8 d in transcription of mesenchymal markers and downregulation of epithelial ones compared with unstimulated controls, which were confirmed by immunofluorescence. Periodontal pathogens also caused a significant increase in level of all cytokines investigated which could be involved in EMT-induction and Snail activation. Exposure of cells to the bacteria increased migration and the rate of wound closure. Downregulation of epithelial markers also resulted in a significant decrease in impedance resistance of cell monolayers to passage of electrical current. These results suggested that EMT was likely induced in OSCC cells in response to stimulation by periodontal pathogens.

ARTICLE HISTORY

Received 12 December 2016
Revised 27 March 2017
Accepted 19 April 2017

KEYWORDS



cadherins; cytokines; invasiveness; migration; mesenchymal cells; neoplasm metastasis; oral keratinocytes

Introduction

Oral cancer is a life-threatening condition if it is not diagnosed and treated in its early stages.¹ Epidemiological data indicates that oral cancer is the nineteenth most common cancer causing mortality in the UK, with oral squamous cell carcinoma (OSCC) being the predominant form accounting for over 90% of oral malignancy cases.^{2,3} OSCC has around 40–50% 5-year survival rates due to recurrence and metastasis.⁴ The invasiveness of cancer cells, has been attributed, in part, to a process called epithelial-mesenchymal transition (EMT).^{5–7} Accordingly, the expression of certain epithelial structural and adhesion molecules is lost while the expression of mesenchymal cell molecules is increased, consequently giving epithelial cells a mesenchymal-like phenotype, at least to some extent.^{6,8,9}

Persistent chronic inflammation due to microbial challenge may favor EMT-induction in various organs

including lungs, liver, and intestine¹⁰ and recent data indicates that infection by the periodontal pathogens *P. gingivalis* and *F. nucleatum* may drive the inflammatory host response and also increase OSCC invasiveness.^{11,12} Oral epithelium responds to the presence of periodontal pathogens by secreting chemokines and cytokines such as transforming growth factor- β 1 (TGF- β 1)¹³ epidermal growth factor¹⁴ and tumor necrosis factor- α (TNF- α)^{15–17} which have been suggested to trigger the onset of EMT either independently or synergistically.^{13,18,19} EGF appears to induce EMT by increasing expression of the transcriptional factor Twist which regulates cell differentiation and lineage definition.²⁰ EMT has been suggested to be responsible for the upregulation of vimentin, a mesenchymal intermediate filament protein, as well as downregulation of the epithelial attachment protein E-cadherin which, in turn could facilitate cell motility

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and compromise epithelial integrity.²¹ A similar mechanism of Twist upregulation is reportedly induced by TNF- α to trigger EMT²² and furthermore, cross talk between Twist-Snail signaling appears to be critical in inducing EMT-like features, increasing anoikis resistance, facilitating cell migration and consequently metastasis.²³⁻²⁵ EMT-related features are usually investigated by using a range of assays including PCR, immunocytochemistry, scratch-wound and transwell migration assays.²⁶⁻²⁹

Recent data from a case-control study has claimed that periodontitis could represent a risk factor for OSCC independent of other risk factors.³⁰ Periodontal pathogens, particularly Gram negative bacteria and their products are well known for their ability to elicit intense chronic inflammatory and immune responses which could trigger EMT. The aim of this work therefore was to examine the potential of *F. nucleatum* and *P. gingivalis* in driving EMT in OSCC *in vitro*.

Materials and methods

Cell culture

The OSCC cell line (H400),³¹ passage numbers 14–35, was cultured in Dulbecco's modified Eagle's medium (DMEM) (SAFC Biosciences, UK) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO₂. Cultures were treated individually with media-only (DMEM and 10% FCS, negative control) or the heat-killed periodontal pathogens, *F. nucleatum* and *P. gingivalis*, at a ratio of 100 bacteria per cell,^{16,32} and 20 μ g/ml *E. coli* LPS (EMT-positive control). Culture supernatants collected from days 1, 5, and 8 were used for further analysis.

Semi-quantitative reverse transcriptase-polymerase chain reaction (sq-RT-PCR) and PCR-array analysis

Total RNA was extracted from cultures using RT lysis buffer (Qiagen, UK) and quantified spectrophotometrically (BioPhotometer plus, Eppendorf, Germany) and visualized in a 1% agarose gel with SYBR Gold. Single stranded cDNA was synthesized from 1 μ g of RNA using the Tetro kit (Bioline, UK) or the RT2 First strand kit (Qiagen, UK).

For sq-RT-PCR cDNA templates were amplified using a thermal cycler (Mastercycler, Eppendorf, Germany) using selected primers (Table 1). PCR products were visualized following separation in 1.5% agarose gels supplemented with ethidium bromide (10 mg/ml) (Sigma, UK). Images were captured using GeneSnap software (Syngene, USA), and analyzed using GeneTools software (Syngene, USA). Relative levels of PCR products were calculated and normalized against the housekeeping gene, GAPDH. All analyses were performed twice and in duplicate for each experiment.

For PCR array analysis, the cDNA template was combined with RT2 SYBER Green Mastermix (Qiagen, UK) before dispensing into the PCR-array plate (96-well format) (Qiagen, UK). Plates were loaded into a thermal cycler (ROCHE, Switzerland). Cycling conditions were 1 cycle at 95°C for 10 min, followed by 45 cycles at 95°C for 15 sec, then terminated by incubation at 60°C for 1 min. Data were collected by LightCycler 480 software and analyzed using the Qiagen online tool (www.SABiosciences.com/pcrarraydataanalysis.php).

TGF- β 1, TNF- α and EGF ELISA

Levels of TGF- β 1, TNF- α and EGF in culture supernatants were assayed 3 times, in triplicate for each time

Table 1. Details of genes studied, primer sequences and semi-quantitative RT-PCR conditions. Accession numbers were obtained from GenBank (T_m: Melting temperature).

wGene name	Gene symbol	Accession number	Sequence (5' → 3')	T _m (°C)	Product (bp)	Number of cycles
Tumor necrosis factor-α	TNF- α	NM_000594.2	F-AAG AAT TCA AAC TGG GGC CT R-GGC TAC ATG GGA ACA GCC TA	60	402	38
Epidermal growth factor	EGF	NM_001963	F-CTTGGGAGCCTGAGCAGAAA R-TGGTTGTGGTCTGAAGCTG	60	521	32
E-cadherin	CDH1	NM_004360	F-CAAGTGCCTGCTTTTGATGA R-GCTTGAAGTCCGAAAAATC	60	339	34
Vimentin	VIM	NM_003380.3	F-CGGGAGAAATTGCAGGAGGA R-ACGAAGGTGACGAGCCATTT	60	588	31
Snail-1	SNAI1	NM_005985.3	F-CGGACCCACACTGGCGAGAAG R-CAGCTGCCCTCCCTCCACAGA	60	433	30
Twist family bHLH transcription factor 1	TWIST1	NM_000474	F-AGACCTAGATGTCATTGTTCCAGA R-CAGGCCAGTTTGATCCCACT	60	454	28

point, using ELISA kits (Quantikine ELISA Kit, R&D Systems, USA) following the manufacturer's instructions.

Scratch wound assay

H400 cells were cultured to confluence in 6-well plates and either treated with media only or bacterial components for 8 d. The epithelial sheet was disrupted to produce a scratch-wound using a sterile disposable plastic pipette tip of 10 μm diameter (Fig 3A) and rinsed with phosphate buffered saline (PBS) before media replenishment.³³ Images were captured immediately after scratching (0 hr) using phase contrast microscopy (Primo Vert, Zeiss, Germany), and at 12 and 24 hr. Images were analyzed using Java-based ImageJ³⁴ and the distance measured at 10 points, using a superimposed grid, between the wound edges. Three images were taken at each time-point and data were averaged. The experiments were repeated 3 times and performed in triplicate for each time point.

Transwell migration assay

Cells (5×10^4) were treated with media only or bacterial components for up to 8 d. Cultures were trypsinized and seeded in 200 μl FCS-free media on the upper compartment of tissue culture insert (Greiner Bio-One, Germany) assembled in the 24-well plate. Media supplemented with FCS (700 μl) were added to the bottom chamber of each well. Cultures were incubated at 37°C and 5% CO₂ for 12 hr. Non-migrated cells in the upper compartment of the membrane were removed using a cotton swab; cells on the lower surface of the membrane were fixed with 4% paraformaldehyde for 15 min followed by Giemsa staining (Sigma, UK).³⁵ Cell counts were performed on 4 random fields of view using phase contrast microscopy (Primo Vert, Zeiss, Germany). All experiments were performed 3 times and in triplicate.

Immunofluorescence and immunohistochemistry analysis

H400 cells were cultured on sterilized glass coverslips (22 \times 22 mm) for 8 d. Cultures were stimulated separately, for 8 days, either with heat-killed *F. nucleatum*, *P. gingivalis* or *E. coli* LPS (20 $\mu\text{g}/\text{ml}$), the control comprised media alone. Cultures were prepared for immunofluorescence and immunohistochemical staining by fixing in 4% paraformaldehyde for 15 min. Cells were permeabilized with 0.25% Triton X-100 in PBS (10 min). Non-specific binding of antibodies was blocked by incubating samples for 30 min with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), followed

by incubation with primary antibodies (1:100) for Anti-E-cadherin (rabbit polyclonal IgG), Anti-vimentin (mouse monoclonal IgG) (Santa Cruz, USA), and Anti-Snail (rabbit polyclonal IgG) (Abcam, UK) overnight at 4°C. For immunofluorescence, secondary antibodies were applied for 1 hr at room temperature. Antibodies used were FITC-conjugated (bovine anti-rabbit) and TRITC-conjugated (goat anti-mouse) for immunofluorescence, both antibodies were from Santa Cruz (USA). Cell nuclei were stained with DAPI (Sigma, UK) and images captured using a confocal microscope (Zeiss, Germany). Analysis was performed on 5 random fields, clusters of cells expressing vimentin were selected, in 3 slides for each group to determine the number of vimentin positive cells compared with total cell number.

For Snail staining, biotin-conjugated (mouse anti-rabbit) (Abcam, UK) was used and counterstained with Mayer's hematoxylin. Slides were viewed by using an inverted microscope (Primo Vert, Zeiss, Germany). A total of 4 random fields from each sample were used to calculate the percent of Snail-positive cells in the fields examined. Both assays were repeated 3 times and performed in triplicate.

Transepithelial electrical resistance (TEER)

This assay was developed to examine integrity of epithelial monolayers *in vitro* by measuring their resistance to the passage of an electric current. Normal barrier function is characterized by increased electrical impedance and the opposite is applied to compromised epithelial layers. Tissue culture inserts with 0.4 μm pore membrane (Greiner bio-one, Germany) were inserted in 24-well plates and seeded with H400 cells (7×10^4) in the upper chamber.³⁶ Two-hundred microliters of media was applied in the upper chamber and 500 μl in the lower compartment. Cultures were exposed separately to media only (control), heat-killed *F. nucleatum* and *P. gingivalis* or *E. coli* LPS (20 $\mu\text{g}/\text{ml}$). Measuring transepithelial electrical resistance (TEER) started at a point where stable recordings were obtained from the culture using an AC-based ohmmeter (EVOM2, USA). The experiment was repeated 3 times and performed in triplicate, TEER measurements were recorded in (Ω) after subtracting the background resistance of the membrane only out of each read out to obtain the resistant of the epithelial layer.

Statistical analysis

Results are expressed as mean \pm one standard deviation. Analyses were performed using one-way ANOVA between multiple groups and *post-hoc* test to compare

differences. Statistically significant results were considered for P values < 0.05 .

Results

Analysis of EMT-associated gene and protein expression in H400 cultures exposed to bacterial components

Analysis using sq-RT-PCR demonstrated that exposure of H400 cells to heat-killed periodontal pathogens and *E. coli* LPS resulted in gene expression profiles reportedly representative of EMT. Eight days of culture bacterial stimulation resulted in upregulation of vimentin, Snail, and Twist (~6-fold upregulation, $P < 0.001$, Fig. 1). *F. nucleatum* exposure stimulated the highest increase in gene expression of these molecules compared with the other 2 bacterial stimulants. Transcript levels of the epithelial adhesion molecule, E-cadherin, was downregulated following exposure to bacterial stimulation by up to 6-fold ($P < 0.001$) relative to controls. Significant upregulation of several cytokines and growth factors was also detected. EGF transcription levels were increased up to

6-fold ($P < 0.001$), while TNF- α and TGF- β 1 showed a ~2-fold upregulation at all time-points compared with controls (Fig 2D, E, F). The highest changes detected were following 8-days of stimulation. Further analysis of samples for this time-point using PCR-arrays demonstrated a comparable pattern of expression for these genes. Moreover; PCR-array analysis indicated changes had occurred to several other EMT-related indicators and hence supported the induction of this phenotype. These included genes expression changes in mesenchymal, epithelial, transcriptional factors, cytokines, signaling pathways and enzymes (Table 2).

Levels of TGF- β 1, EGF and TNF- α (Fig. 2) in the supernatant of H400 cultures stimulated with bacteria increased significantly ($P < 0.05$) over the 8-day culture period. At day 1, only *P. gingivalis* exposure did not produce a significant increase in levels of these cytokines secreted by the H400 cultures. In general, periodontal pathogens caused 1–10-fold increases, relative to controls, in the levels of TGF- β 1, EGF and TNF- α over the experimental period.

Analysis of immunofluorescence images (Fig. 3A) showed an increase in the percentage of vimentin positive

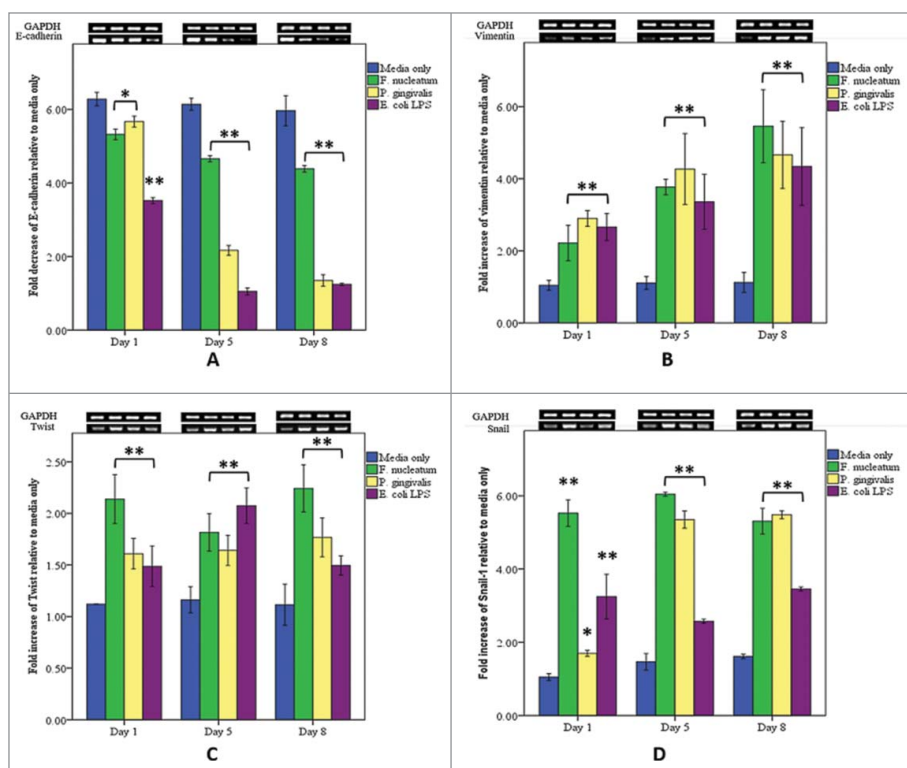


Figure 1. Exposure of H400 cells to heat-killed *F. nucleatum*, *P. gingivalis*, and 20 μ g/ml *E. coli* LPS produced changes in EMT-related indicators. Gel images and densitometric analysis showing fold changes relative to highest or lowest levels as determined for selected EMT-related genes using semi-quantitative RT-PCR analysis. *F. nucleatum* and *P. gingivalis* produced significant changes in (A) E-cadherin level after 24 hr cell stimulation and continued till day 8 (~5-fold). Other EMT-related genes [(B) vimentin, (C) Twist, and (D) Snail-1] were upregulated up to 5-fold compared with unstimulated control in response to periodontal pathogens from days 1–8 (* = $P < 0.05$, ** = $P < 0.001$).

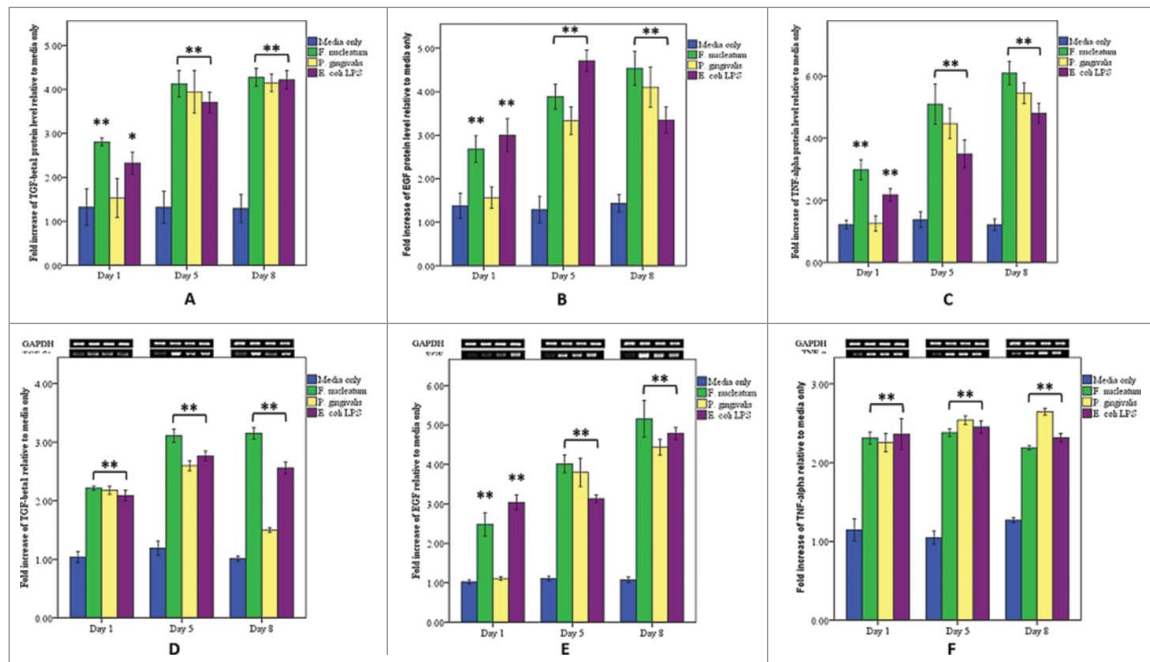


Figure 2. H400 cells were treated with media only (DMEM supplemented with 10% FCS), or heat inactivated periodontal pathogens *F. nucleatum* and *P. gingivalis* (100 bacteria per cell), and 20 $\mu\text{g/ml}$ *E. coli* LPS over 8 d. Supernatant collected from each group after 1, 5, and 8 d and assayed using ELISA for (A) TGF- β 1 which significantly increased with *F. nucleatum* and *E. coli* LPS (\sim 2 folds) at day 1, while *P. gingivalis* showed no difference compared with the control. Day 5 and 8 data showing significant upregulation for all test groups (\sim 6-folds). (B) EGF and (C) TNF- α , results showing similar pattern of stimulation to that of TGF- β 1 in which *P. gingivalis* did not produce any statistical difference in the amount of EGF and TNF- α released in comparison with the control. At day 5 and 8 all bacterial groups significantly upregulated the level of both cytokines in the supernatant. The same picture is applied to the increase in gene transcription for the same cytokines (D, E, F), the only exception is the significant increased transcription of TNF- α associated with *P. gingivalis* after 24 h (* = $P < 0.05$, ** = $P < 0.001$).

stained H400 cells following bacterial exposure ($P < 0.05$) at 8-days culture. This change was also accompanied by a decrease of E-cadherin expression on the cell membrane of stimulated cells when compared with controls and was associated with co-existence of vimentin positive staining (Fig 4A). Consistent with these findings, immunohistochemistry analysis (Fig. 4B) indicated a significant increase ($P < 0.05$) in the proportion of Snail-positive cells, following the same period of stimulation as compared with the control.

Epithelial integrity and migratory responses in H400 cultures exposed to bacteria

As EMT is associated with a decrease of expression of molecules involved in cellular attachment, it is suggested that this is a contributor to cell dispersion. *in vitro* TEER was performed to assess H400 culture integrity. Figure 4C shows that resistance in H400 monolayers (cultured in the presence of bacterial components) was significantly lower than controls ($P < 0.05$) at day 4 following exposure to *F. nucleatum*, *P. gingivalis*, and *E. coli* LPS. All bacterial exposures from day 4 till 8 exhibited

Table 2. EMT PCR-array fold changes in transcription of EMT-related genes following exposure to bacterial components. Table was generated by using Qiagen online analysis tool (www.SABioSciences.com/pcrarraydataanalysis.php).

Gene name	Gene Symbol	Fold changes relative to control		
		<i>F. nucleatum</i>	<i>P. gingivalis</i>	<i>E. coli</i> LPS
Transforming growth factor β 1	TGF- β 1	2.8	2.2	1.6
Bone Morphogenic protein 7	BMP-7	2.5	4.7	6
Glycogen synthase kinase 3 β	GSK3 β	1.6	4.6	2.7
Jagged 1	JAG1	4.5	2	1.3
Notch 1	NOTCH1	6.9	2.1	1.9
Snail-1	SNAI1	4.3	6.3	1.9
Slug	SNAI2	1.5	3.1	1.6
Zinc finger E-box binding homeobox 1	ZEB1	3.2	7.8	2.9
E-cadherin	CDH1	-5.4	-4.3	-3.5
β -Catenin	CTNNB1	-2	-3.1	-1
Cytokeratin 14	KRT14	-2.1	-2.5	-1.1
N-cadherin	CDH2	6.3	2.4	5.7
Vimentin	VIM	5.5	4.8	2.6
Matrix metalloproteinases-2	MMP2	2.4	2	3.1
Matrix metalloproteinases-9	MMP9	6.5	2.1	2.4
Smad family member 1	SMAD1	2.4	1.8	2.4
Twist family bHLH transcription factor 1	TWIST1	2	4	6.6

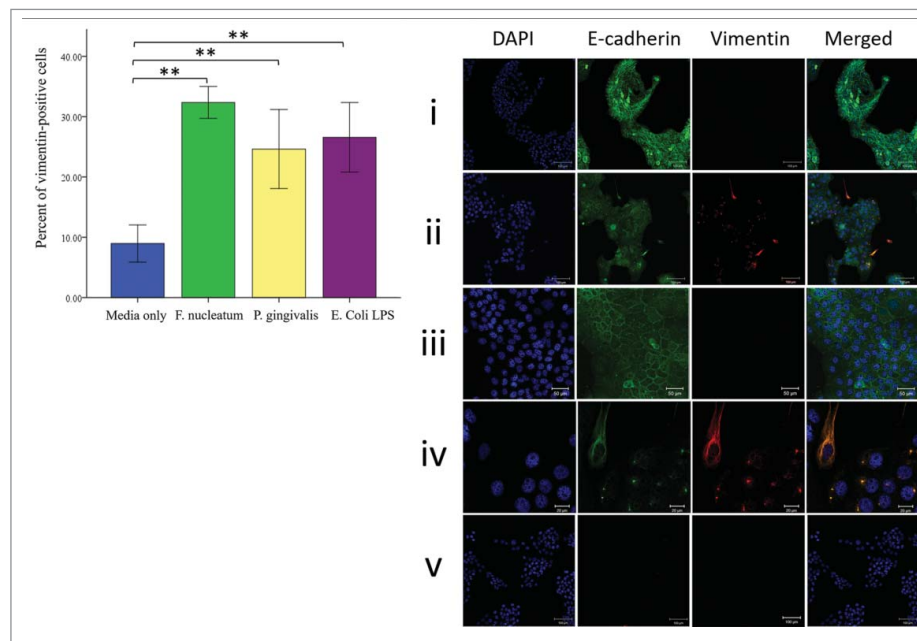


Figure 3. Periodontal bacteria modulate vimentin and E-cadherin expression in H400 cells after 8-days culture. (A) Cells with vimentin positive staining were counted and expressed as a percentage. Analysis indicated a significant increase between cells stimulated with bacteria and unstimulated control in terms of vimentin expression. (B) Immunofluorescence staining indicates that *F. nucleatum* and *P. gingivalis* induce expression of vimentin (red) (ii) when compared with the control group treated with media only (i). Scale bars = 100 μm . Higher magnification showing that unstimulated control (iii) maintained normal E-cadherin distribution and negatively expressed vimentin. Scale bar = 50 μm , while stimulated cultures (iv) showed presence of vimentin-positive cells which either exhibit mesenchymal-like morphology and retained some characteristics of their parental origin by expressing E-cadherin or cluster of epithelial cells simultaneously expressing vimentin with downregulation of E-cadherin from periphery of cells. Scale bars = 20 μm . Negative controls (cultures treated with secondary antibodies only) were included to exclude possibility of unspecific staining (v). (** = $P < 0.001$).

significantly reduced TEER, indicating that integrity of epithelial sheets in the cultures was compromised after exposure to periodontal pathogens. Consistent with these findings, culture images (Fig 4D) showed that cells treated with bacteria showed scattering associated with morphological changes resembling that of fibroblasts, while unstimulated cultures maintained a classical epithelial cobble-stone appearance.

Increased cell locomotion is regarded as an indicator of acquisition of a mesenchymal phenotype. H400 cells previously exposed to periodontal pathogens over 8 d were used in the transwell migration assay. Data (Fig 5A) indicated that periodontal pathogens increased migration rates in H400 cells ($P < 0.001$) as compared with media-only treated cells. To explore further the migratory ability, a scratch-wound assay was undertaken on H400 confluent monolayers exposed to the periodontal pathogens (8 days). Wounding was repeated many times on several samples, stimulated and control, and carefully selected to ensure that all of them had the same initial gap width (500 μm). This approach was used to eliminate any variation in closure rate due to difference in distance between wounded edges. Measurements at 12 hr did not show any significant difference in gap closures between test and control groups. However, at

24 hr, cultures exposed to bacteria exhibited greater gap closures ($P < 0.05$) when compared with controls (Fig 5B, C).

Discussion

The poor prognosis of OSCC represents a significant health challenge despite advances in diagnostic and therapeutic measures mainly due to the high recurrence and metastasis rates.³ The oral tissues are exposed to a relatively large population of potentially pathogenic bacteria, in particular, Gram negative anaerobes.³⁷ Recent research has highlighted a potential involvement of periodontal pathogens, *F. nucleatum* and *P. gingivalis*, in increased aggressiveness of OSCC.^{11,12} Samples from highly malignant gingival squamous cell carcinoma showed an abundant presence of *P. gingivalis*,³⁸ however their role and reason for the observed association remain unclear.

Recent studies on EMT indicated that this process provides a potential mechanism to explain some of the required steps leading to metastasis.^{5,7} In the current work, we investigated the possible effect of periodontal pathogens in triggering EMT in OSCCs *in vitro*. The transition of cell phenotype by EMT requires activation

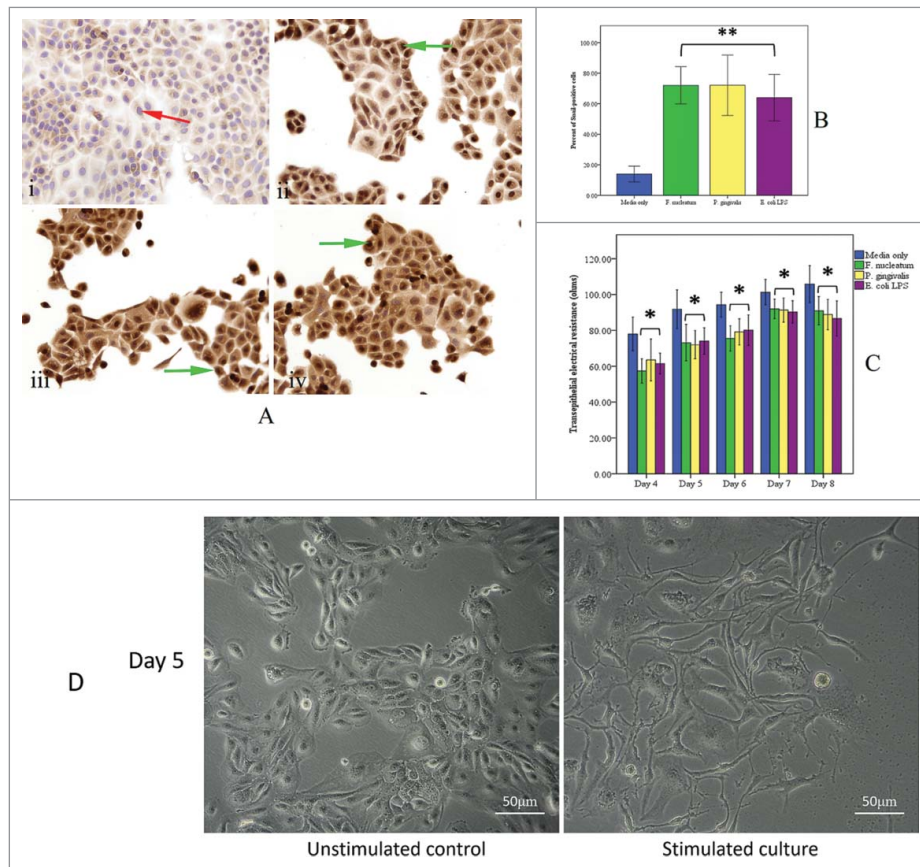


Figure 4. Stimulation of H400 cells with periodontal bacteria resulted in significant increase in Snail-positive cells. (A) Immunocytochemical staining of H400 cell, growing on 22 × 22 mm coverslips, showing increased Snail expression. Activated cells manifested by dark brown discoloration of the nucleus and/or cytoplasm (green arrow), while cells treated with media only mostly exhibit dark blue nucleus (red arrow). Scale bars are (50 μm) (B) Analysis of percent of Snail-positive cells showing that *P. gingivalis* and *F. nucleatum* elicited the higher response (up to 70%) when compare with unstimulated control. (C) H400 cells were cultured on a 0.4 μm pore membrane insert and stimulated with periodontal pathogens. Resistance levels were stabilized in all groups after 4 days, from this point TEER measurements were recorded up to day 8. Analysis of data showed that resistance in response to bacterial stimulation was significantly decreased from day 4 to 8 in comparison with the media control group. Following 5 d of exposure to periodontal pathogens, cells in stimulated cultures exhibited fibroblast-like morphology while unstimulated control maintained normal epithelial sheet architecture (D). Scale bars = 50 μm. (* = P < 0.05, ** = P < 0.001).

of certain inducer molecules of which Snail and Twist appear to be relevant candidates.^{20,23} Snail is a zinc-finger transcription factor that increases in activity during inflammation and is a well characterized regulator of E-cadherin expression.³⁹ In addition, increased Snail expression has been reported in recurrent and metastatic OSCCs, in comparison with cancer cells from the primary tumor.²⁴ PCR data showed that both transcriptional factors were increased <6-fold over 8 d of culture following bacterial stimulation. Notably the activation of these transcriptional factors requires activation of signaling pathways by a range of cytokines. Consistent with this was data from the EMT PCR-array which indicated increased transcription of essential EMT-signaling pathways GSK3β, JAG1, and NOTCH. However, the changes in signaling pathways were not equally significant among bacterial stimuli investigated (Table 2). This may provide

insight into the differential activation of certain pathways by different bacteria. While PCR-array data was consistent with our sq-RT-PCR findings it also identified further changes: upregulation of MMP-2, -3 and -9 compared with controls. These molecules are involved in the breakdown of the basement membrane, which could facilitate tumor metastasis.^{8,9}

Previous work by Milward and coworkers¹⁶ showed that presence of non-viable periodontopathogens in the culture of epithelial cells increase transcription TNF-α, IL-1β, and IL-8. In this study, TGF-β1, EGF and TNF-α were initially selected for investigation as they share a common EMT-signaling pathway which results in stabilization and activation of Snail.^{39,40} We originally hypothesized that these factors may drive the EMT phenotype chronically due to local inflammatory events. Assaying levels of TGF-β1, EGF and TNF-α in the

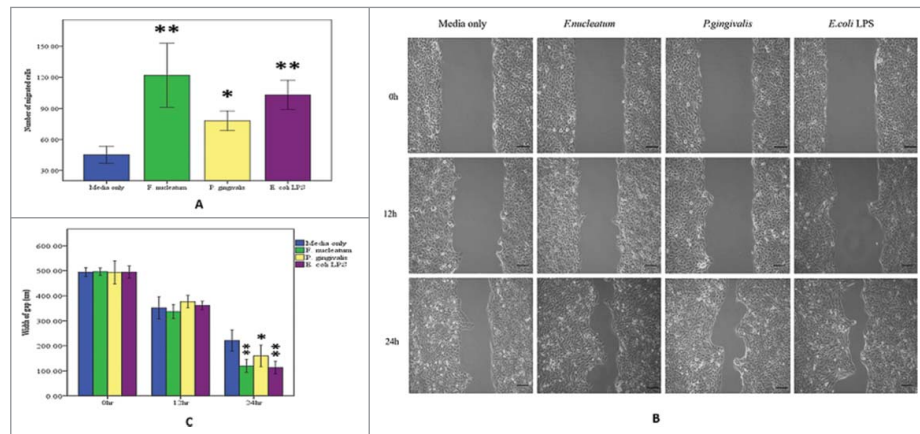


Figure 5. (A) Analysis of transwell migration assay for H400 cells stimulated over 8 d with periodontal pathogens. Data demonstrated that a higher number of cells had migrated through the membrane in comparison with control. Confluent monolayers of H400 cells were disrupted by scratching with a 10 μm pipette tip following 8 d of exposure to heat-inactivated *F. nucleatum* and *P. gingivalis* and 20 $\mu\text{g/ml}$ *E. coli* LPS. (B) Images were captured immediately following scratching and then after 12 and 24 hr culture. Scale bars are 100 μm (C) Measurements of the wound width at 12 hr indicated no significant difference, however at 24 hr scratch closure was significantly increased in all cultures exposed to bacterial components in comparison with unstimulated control. Data is shown as mean + SD. Experiments were performed in triplicate. (* = $P < 0.05$, ** = $P < 0.001$).

supernatant from H400 cultures showed that bacterial components increased production of these cytokines although this was not evident for *P. gingivalis* 24 hr post-exposure. Notably these findings were consistent with previous reports indicating that *P. gingivalis* had a weaker effect, compared with *E. coli* LPS, in stimulating TNF- α over a 24 hr period both *in vivo* and *in vitro*.⁴¹

Evidence for EMT triggering requires detection of certain changes which have been described previously as major criteria including downregulation of adhesion molecules, increased migratory ability, and expression of new mesenchymal markers.⁴² Transcription of E-cadherin, the central protein of adherens junctions involved in cell attachment, is reportedly decreased significantly following increased transcription of Snail,⁴³ and Twist.²⁵ E-cadherin downregulation was also accompanied by upregulation of N-cadherin (Table 2) and supports the switching from epithelial into mesenchymal cadherins which is considered one of the main features of EMT.^{8,9} Snail activity, and E-cadherin suppression, stimulated by periodontal pathogens, was confirmed here by immunohistochemical staining. Snail activation after 8 d of bacterial stimulation showed that 70% of cells were activated by *F. nucleatum* and *P. gingivalis*. Immunofluorescence staining and PCR data showed a simultaneous upregulation of vimentin expression after stimulation with bacterial products, further supporting the process of EMT induction in the H400 OSCC line *in vitro*.⁴⁴ Up-regulation of vimentin has been reported to be induced by EGF, which is a key regulator of proliferation and migration of cells.⁴⁵ Transitioned cells co-expressing vimentin and E-cadherin cells were elongated associated with E-

cadherin internalization. Other cells still forming epithelial clusters with E-cadherin distributed around the cell membrane; however, vimentin could be detected in their cytoplasm. (Fig. 3B).

Exposure to periodontal pathogens resulted in a higher rate of closure of the scratch-wounds inflicted on H400 monolayer cultures. In the present study, the migratory ability of the cells was investigated by using simple Boyden or transwell-migration assays which can provide thorough information about cell migration through a barrier in response to a chemo-attractant agent.⁴⁶ In addition, the wound-healing assay which is simple, cheap and well-developed method to measure cell migration *in vitro*⁴⁷ was used to further confirm the increased motility of epithelial cells in response to stimuli. Our results indicated that wound closure was consistent with an increase in numbers of migrated cells as supported by the transwell migration assay (Fig. 5A). The same pattern of migration rates was detected in both assays for *F. nucleatum* and *P. gingivalis*. These findings are also consistent with the PCR and immunofluorescence data demonstrating downregulation of E-cadherin as well as the TEER results showing decreased epithelial integrity which would potentially enable cell motility. The decrease in electric resistance of the cultures to the passage of a current correlated with the loss of cellular attachment and changes in cellular morphology as observed with light microscopy (Fig 4D). The increased vimentin expression was a potential indicator for the presence of cell populations with high migratory capabilities as well as populations usually associated with cancers.⁴⁸ These data were in agreement with previous

results²¹ which demonstrated increased cell motility was correlated with vimentin expression and associated with E-cadherin downregulation. In addition, compromised epithelial integrity may suggest onset of EMT due to decrease in cellular junctions.⁶ Notably this occurs because of E-cadherin and other epithelial adherence protein downregulation. This feature was also supported by the decreased electrical impedance following exposure to bacteria.

In conclusion, this study suggested that the periodontal pathogens elicit changes in H400 cells, OSCC cell line, at the molecular, structural and behavioral levels which are potentially indicative of EMT. Further investigation on the potential of periodontal pathogens to induce EMT in different cell lines and primary cells are likely to complement our findings and provide additional information on the clinical importance of EMT induction, and their relation to compromised integrity of epithelial tissue, invasion and metastasis.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This study was sponsored by Iraqi Ministry of Higher Education and Scientific Research. GL acknowledges support from EPSRC grant EP/M023869/1.

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