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Regulation of E-cadherin and TGF-β3 expression by CD24 in cultured oral epithelial cells

P. Ye *, M.A. Nadkarni, N. Hunter

Institute of Dental Research, Westmead Millennium Institute and Westmead Centre for Oral Health, Westmead Hospital Westmead, NSW, Australia

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Abstract

We previously reported evidence that patients with periodontitis have serum antibodies to oral Gram positive bacteria that are cross-reactive with epithelial antigens, including CD24. High level expression of CD24 was confined to the reactive periodontal epithelium and inflamed gingival attachment. As a model for the reactive epithelium of chronic periodontitis, H413 epithelial cells derived from a human oral squamous cell carcinoma were cloned and lines expressing high levels of CD24 were selected. RNA interference protocols were designed to determine if CD24 could modulate intercellular interactions and regulate the biology of these epithelial cells. Knock-down of CD24 protein was demonstrated by Western blot and flow cytometry. The level of mRNA for CD24 was reduced 90% by RNAi treatment as assayed by real-time, reverse transcriptase (RT)-PCR. Gene products known to be important in epithelial biology, including E-cadherin and TGF-β3 that were demonstrated to undergo altered expression patterns in the periodontal lesion, were investigated. Down-regulation of CD24 mRNA was associated with reduced *e-cadherin* expression and up-regulated expression of *snail*, *twist*, and *tgf-β3*. The cells were treated with monoclonal antibodies to CD24 to mimic the action of auto-reactive antibodies to CD24 detected in affected patients. Relative to isotype control antibody, stimulation by anti-CD24 antibodies induced up-regulated expression of *e-cadherin* and down-regulation of *tgf-β3* as assessed by real-time RT-PCR. No consistent changes for expression of *β-catenin*, connexins, integrins, *icam-1*, *tgf-β1* or *tgf-β2* were observed. CD24 could play an important role in modulating expression of genes that regulate epithelial differentiation in periodontal disease.

Keywords: CD24; E-cadherin; TGF-β3; Epithelial cell; Auto-antibody; Gene expression; siRNA; Periodontitis

Immunopathological responses to bacterial antigens are considered to be central in the pathogenesis of the destructive lesion of periodontitis that is characterized by profound perturbation of normal epithelial structure. The reactive epithelium associated with inflammatory periodontal disease is derived from the specialized epithelium of the gingival attachment to the tooth surface [1] and possibly, from developmental remnants that persist in this location [2]. This so-called reactive pocket epithelium has a number of features that discriminate it from other stratified squamous epithelia. These include cytokeratin [3] and involucrin [4] expression profiles that do not support a typical pattern of terminal differentiation. Further, there is evidence for reduced expression of E-cadherin critically required for maintenance of adherens junctions between epithelial cells, of connexins that mediate intercellular communication in epithelia through gap junctions and of perturbation of F-actin filament structure [4]. The reactive epithelium also strongly expresses the TGF-β3 isoform [5] normally expressed by connective tissue cells [6]. These particular expression profiles have implications for both intercellular contact and communication with the extracellular matrix through respective integrin-mediated adhesions [7].

Recently we reported auto-reactive antibodies cross-reactive with antigens of Gram positive bacteria located in the pocket polymicrobial plaque in patients with periodontal disease [8]. These antibodies recognize a number of antigens including CD24 [9] that was demonstrated to be

* Corresponding author. Fax: +61 2 9845 7599.
E-mail address: pingy@dental.wsahs.nsw.gov.au (P. Ye).

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selectively expressed at high levels by the reactive epithelium of the periodontitis lesion [9]. Assay of patient serum antibodies reactive with CD24 indicated a positive relationship between the titre to CD24 and an improved disease status [9]. CD24 is a heavily glycosylated peptide implicated in haematogenous metastasis of carcinomas [10] and local retention of auto-reactive T cells [11]. CD24 is anchored by phosphatidylinositol linkage to lipid rafts within the cell membrane [12] and available literature [13] indicates that CD24 mediates expression of cell adhesion molecules in B lymphocytes. Evidence for a signaling function for the CD24 receptor comes from the regulation of apoptosis by monoclonal antibodies reactive with CD24 in B cell precursors [13]. CD24 has been demonstrated to be a ligand for P-selectin and was recently shown to be a regulator of the chemokine CXCR4 [14]. The objective of the present study was to develop preliminary findings by investigating the role of CD24 in regulating epithelial biology relevant to the lesion of chronic periodontitis.

Materials and methods

Oral epithelial cultures and clones. The epithelial cell line H413 that was derived from a human oral squamous cell carcinoma [15], displayed a stratified epithelial cell morphology in culture. The cells were cultured at 37 °C with 5% CO_2 in Minimum Essential Medium Eagle's medium (MEME, Joklik modification) and supplemented with 10% fetal calf serum (FCS, CSL Limited, Victoria, Australia) and penicillin/streptomycin (100 IU/ml, ICN Biomedicals, Inc., Sydney, Australia). Cell cultures were harvested using 0.05% trypsin/EDTA in PBS and sub-cultured every 3 days. H413 cell clonal lines were established using a limit dilution method. Single cells that grew as a symmetrical colony in 96 well plates were selected for subculture and expanded into 24-well plates, 25 cm^2 flasks, and 75 cm^2 flasks (Sarstedt Australia Pty Ltd. South Australia). Cloned lines expressing high levels of CD24 were selected by screening using analytical flow cytometry. H413 clone-1 cells exhibiting both characteristic epithelial morphology and high CD24 expression were chosen for this study.

CD24 siRNA preparation and transfection of H413 clone-1 cells. CD24 target-specific siRNA (GenBank Accession No. M58664) was designed using two on-line design tools http://imgenex.c.topica.com/ and http://bioit.dbi.udel.edu/rnai/ [16]. Four target sequences were selected (Table 1) and the double-stranded oligonucleotides were designed for the GeneSuppressorNeo system (IMGENEX Corporation, San Diego, CA, USA) as per the manufacturer's instructions (version 071404) and were synthesized by Invitrogen (Australia). The siRNA inserts were cloned into *Sal*1 and *Xba*1 sites (Fig. 1) according to the manufacturer's instruction. A control ligation (without insert) was also performed. The ligated plasmid DNA was transferred into *Escherichia coli* strain XL1-Blue using an electroporation protocol routinely used in our laboratory (2.5 kV, 25 μF, time constant 4.6 ms) using a Gene Pulser apparatus (Bio-Rad). After transformation cells were then cultured in LB medium containing 25 μg/ml of kanamycin. Plasmid DNAs were extracted by Wizard plus sv minipreps DNA purification system (Promega) and detected by electrophoresis in 1% agarose gel with ethidium bromide (0.5 μg/ml) and visualized under UV illumination. The plasmid DNAs were stored at −20 °C until used. Before transfection, H413 clone-1 cells were passaged using fresh medium without antibiotics. For electroporation the cells were detached by trypsinization, re-suspended at 3×10^6/ml in fresh serum-free medium without antibiotics and electroporated with two pulses at 0.25 kV, 500 μF, and 18–21 ms time constant using a Gene Pulser apparatus (Bio-Rad). Between 16 h and 48 h after electroporation, 200 μg/ml G418 (Geneticin, Sigma) was added to select for the Neo resistance gene (*NeoR*).

Table 1
Target sequences of CD24 siRNA candidates

	Sequences	Location	GC%
Insert-1	5′-TCCAACTAATGCCACCACCAA-3′	202–223	47
Insert-2	5′-GTCTCTTCGTGGTCTCACTC-3′	256–276	55
Insert-3	5′-GGAACTTCCAGGTGTTACTGT-3′	752–773	47
Insert-4	5′-GCATCCTGAGCAACTCTTGAT-3′	978–999	47

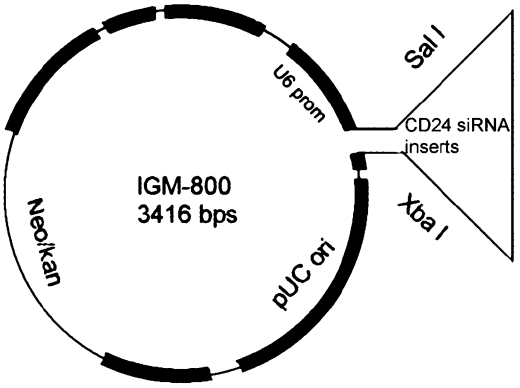


Fig. 1. Schematic diagram of the pSuppressorNeo plasmid. The CD24 siRNA insert was cloned into *Sal*1 and *Xba*1 sites.

Immuno-detection of CD24 in siRNA-treated cells. For Western blotting, proteins extracted from H413 clone-1 cells were separated on standard SDS-PAGE with 12% polyacrylamide mini-gels, transferred to nitrocellulose membranes (Bio-Rad, Sydney, Australia) and blocked with 3% bovine serum albumin in Tris buffered salts solution, pH 7.4, (TBS) overnight. Blotted antigens were incubated with monoclonal mouse anti-human CD24 (DAKO, Denmark) diluted 1:800 in 0.05% Tween 20/TBS for 1.5 h, washed and incubated with alkaline phosphatase (AP)-conjugated second antibody (goat anti-mouse IgG-DAKO) diluted 1:1500 in Tween/TBS for 1.5 h. CD24-bound antibody was visualized with AP substrate (Bio-Rad).

For immunofluorescence staining, cells were washed in cold phosphate buffered saline (PBS) and collected by trypsinization. After centrifugation, cells were resuspended in cold PBS containing 2% fetal calf serum (FCS) with 0.1% sodium azide, in 75 mm tubes (Becton Dickinson), vortexed, and incubated with monoclonal mouse anti-human CD24 (1:100, DAKO, Denmark) on ice for 30 min. After three washes in 2% FCS/PBS, with 0.1% sodium azide, FITC-conjugated rabbit anti-mouse IgG (1:50) was added, mixed in, and incubated in the dark on ice for 30 min. After three final washes, aliquots of at least 20,000 cells in 300 μl of 2% FCS/PBS with 0.1% sodium azide were examined using a FACScan and analyzed using CellQuest software (Becton Dickinson, Franklin Lakes, N.J.). Each assay was repeated in three separate experiments.

Cell treatment by CD24 peptide antibodies. Sub-confluent H413 clone-1 cells (25 cm^2 flask containing 5×10^5 cells) were incubated with 5 μg/ml of CD24 mouse monoclonal (ALB9) peptide antibody (IgG1, Abcam Ltd., Cambridge, UK) which recognized a short non-glycosylated peptide sequence close to the site of GPI linkage of the peptide core of the cluster-w4/CD24 antigen [17]; or with an IgG1 negative control (DAKO, Denmark), respectively. Cells were cultured overnight to reach 80% confluence and harvested by Trizol reagent (Invitrogen) for isolation of RNA (see below).

RNA extraction. Total RNA was isolated from each test or control sample as follows: normal H413 clone-1 cells, cells transfected with CD24 siRNA, cells transfected with plasmid without insert, cells treated with monoclonal mouse anti-human CD24 peptide (ALB9) antibody, and cells treated with the same isotype IgG1 negative control, using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Briefly, cells were harvested by scraping in PBS, centrifuged, and 1 ml Trizol added to the cell pellet (5×10^6) for homogenization, followed by extraction into chloroform and isopropanol. The RNA pellets were washed in 75% (v/v) ethanol, centrifuged, air dried, and resuspended in an appropriate volume

Table 2
Gene expression patterns assessed in H413 clone-1 epithelial cells using gene arrays

Tube	Gene name	Expected size (bp)	Gene symbol	UniGene	GenBank Accession Nos
1	E-cadherin	191	CDH1	Hs.194657	NM_004360
2	β-catenin	174	CTNNB1	Hs.476018	NM_001904
3	Connexin 43	186	GJA1	Hs.74471	NM_000165
4	Connexin 26	156	GJB2	Hs.524894	NM_004004
5	Integrin-β1	157	ITGB1	Hs.287797	NM_002211
6	Integrin-β6	210	ITGB6	Hs.57664	NM_000888
7	Integrin-α3	109	ITGA3	Hs.265829	NM_002204
8	TGF-β1	91	TGFB1	Hs.1103	NM_000660
9	TGF-β3	128	TGFB3	Hs.2025	NM_003239
10	Snail	106	SNAI1	Hs.48029	NM_005985
11	Twist	162	TWIST1	Hs.66744	NM_000474
12[a]	TGF-β2	197	TGFB2	Hs.133379	NM_003238
13	Integrin aV	153	ITGAV	Hs.436873	NM_002210
14	ICAM-1	181	ICAM1	Hs.515126	NM_000201
15	GAPDH	168	GAPD	Hs.169476	NM_002046

[a]The three genes highlighted were weakly expressed by H413 clone-1 cells.

of DEPC-treated MilliQ water. To monitor plasmid-induced expression of the interferon (IFN) response gene oligoadenylate synthase-1 (*OAS1*) [18,19], RNA from healthy human peripheral blood mononuclear cells (PBMCs) and PBMCs infected with HIV virus was used as negative and positive control, respectively.

Reverse transcriptase (RT)-PCR. The First-Strand cDNAs were synthesized with oligo$(dT)_{20}$ (Invitrogen), 10 mM dNTP (Promega), RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen) and SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol (Invitrogen).

Target genes (Table 2) were selected on the basis of either known modulation in the reactive epithelium of the periodontitis lesion or genes considered to represent key aspects of epithelial structure and function. The panel of integrin genes selected was based on expression patterns reported for oral epithelia [7]. Expression profiling PCR arrays for the target genes were synthesized by SuperArray Bioscience (MD, USA), and amplified in a thermal cycler (FTS-320, Corbett Research, Sydney, Australia) at 95 °C, 15 min; 30 cycles of 95 °C, 30 s; 55 °C, 30 s and 72 °C, 30 s. RT-PCR for the NeoR-gene, CD24 gene and OAS1 gene were carried out at 95 °C, 10 min; 30 cycles of 95 °C, 15 s; 60 °C, 60 s. Each experiment was performed three times.

When the PCR array incubations were complete, 10 μl of each reaction mixture was loaded into separate wells in a 2% agarose gel containing 0.5 μg/ml ethidium bromide in 1 × TAE buffer and a 5 μl of 100 bp DNA ladder was loaded. The images of the gels on a UV Trans Illuminator were captured by GeneSnap with CCD camera and analyzed by Gene Tools' software from GeneGenius (Syngene, MD, USA).

Real-time (TaqMan) RT-PCR for CD24, OAS1, e-cadherin, snail, twist, and tgf-β3. Real-time RT-PCR analyses were performed by TaqMan assays using the ABI PRISM 7700 Sequence Detection System and Software (version 1.6.3) (Applied Biosystems, Inc., Foster City, CA). The primers and probe sets shown in Table 3 were based on reported amplicons [20,21] but were modified using Primer Express software (Perkin Elmer, Foster City, CA) to achieve optimal results. Primers were synthesized by Invitrogen while TaqMan probes (labeled with fluorescent dyes 5′-FAM and TAMRA-3′), TaqMan PCR Core Reagent Kit and TaqMan β-actin Control Reagents were purchased from Applied Biosystems.

Table 3
RT-PCR and real-time RT-PCR sequences of primers and TaqMan probes

Oligo	Sequence 5′ → 3′	Size of PCR product (bp)	T_m (°C)
NeoR-gene[a]			
Forward	GAACTCGTCAAGAAGGCGATAGA	776	58.7
Reverse	GATGGATTGCACGCAGGTT		58.1
CD24[b]			
Forward	CCCACGCAGATTTATTCCAG	254	57.9
Reverse	GACTTCCAGACGCCATTTG		56.5
Probe	TCGTGGTCTCACTCTCTCTTCTGCATCTCTA		67.2
OAS1[c]			
Forward [20]	AGGTGGTAAAGGGTGGCTCC	88	58.9
Reverse	GGTGAGAGGACTGAGGAAGACAA		58.5
Probe	CAGGTCAGCGTCAGATCGGCCTCT		68.6
E-cadherin [21]			
Forward	GAACAGCACGTACACAGCCCT	76	58.9
Reverse	GCAGAAGTGTCCCTGTTCCAG		58.4
Probe	ATCATAGCTACAGACAATGGTTCTCCAGTTGCT		67.2
TGF-β3			
Forward	CCACAACCCTCATCTAATCCTCA	63	58.9
Reverse	CCTGGCCCGGGTTGTC		59.4
Probe	ATGATGATTCCCCCACACCGGCT		68.2
Snail [21]			
Forward	TGCAGGACTCTAATCCAGAGTTTACC	91	60.0
Reverse	GTGGGATGGCTGCCAGC		59.7
Probe	TCCAGCAGCCCTACGACCAGGC		68.4
Twist [21]			
Forward	TGTCCGCGTCCCACTAGC	92	59.4
Reverse	TGTCCATTTTCTCCTTCTCTGGA		58.8
Probe	ACATCTAGGTCTCCGGCCCTGCTGA		68.7

[a] NeoR-gene refer to kit IMG-800 (IMGENEX, San Diego, CA, USA).
[b] CD24 primers were purchased from Sigma.
[c] OAS1 Accession No: NM_016816.

According to the kit protocol, 2 μl of diluted cDNA samples, 200 nM of the probe, and 200 nM primers in a 25 μl final reaction mixture were used. The PCR was initiated by activation of AmpliTaq Gold at 95 °C for 10 min, followed by 40 PCR cycles denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. E-cadherin and β-actin cDNA isolated from un-manipulated H413 clone-1 cells was used for constructing standard curves (2000–2 pg). All TaqMan assays were performed in triplicate and repeated three times.

Data analysis. For captured target genes, RT-PCR profiling images were analyzed using Gene Tools software (GeneGenius Syngene, MD, USA). Normalized values for relative intensity of genes of interest were determined by dividing each of the background-corrected RT-PCR band values by the background-corrected value for the GADPH housekeeping gene. Paired *t*-tests were used to analyze data from at least three consecutive experiments. For real-time TaqMan RT-PCR data, the ratios for target genes compared with β-actin control were markedly asymmetric and therefore the non-parametric Mann–Whitney test was performed. A level of $P < 0.05$ was accepted as statistically significant.

Results

Assessment of siRNA manipulation

Real-time RT-PCR for the expression of the interferon response gene *OAS1* indicated up-regulation by 2.86-fold (mean of three experiments) in viral-infected PBMC compared with PBMC control. In contrast, the mRNA levels for OAS1 in H413 clone-1 cells transfected with plasmid with and without CD24 siRNA averaged 1.1-fold compared with PBMC control. Therefore the short CD24 siRNA did not activate expression of the interferon (IFN) response gene OAS1. Persistence of the transfecting plasmid in electroporated H413 clone-1 cells was confirmed by the expression of *NeoR* (Fig. 2A). Insert-1 siRNA (Table 1) was most effective in suppression of mRNA for CD24 with insert-2 being less effective (Fig. 2B). Insert-3 and insert-4 did not produce detectable suppression of CD24 mRNA levels. By real-time RT-PCR insert-1 siRNA reduced CD24 mRNA by an average of 90% (Fig. 2C).

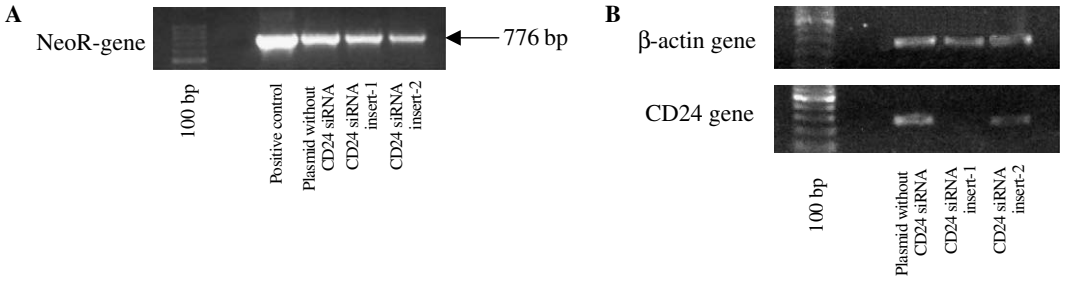
Corresponding reduction at 3 days' culture of CD24 protein was assessed by Western blot (Fig. 3A) and flow cytometry (Fig. 3B).

Experimental manipulation of H413 clone-1 cells by siRNA for CD24

MultiGene-12 Strips were visualized on a 2% agarose gel (Fig. 4A) following RT-PCR for 30 cycles and compared to a control plasmid-only sample. The values were normalized against the constitutively expressed control gene GAPDH and measured using Gene Tools software. A ratio between the level of specific gene expression detected in H413 clone-1 cells transfected with plasmid without a CD24 siRNA insert and cells transfected with plasmid containing the siRNA insert was calculated. The quantitative data for experiments showed significantly reduced E-cadherin mRNA expression ($P < 0.05$) associated with up-regulation of *snail*, *twist* ($P < 0.05$) and *tgf-β3* ($P < 0.01$) by paired *t*-test (Fig. 4C). Real-time quantitative RT-PCR analysis confirmed that CD24 siRNA mediated down-regulation of E-cadherin mRNA (0.32-fold of control; $P < 0.05$) and up-regulation of *snail* (fold change compared with control = 2.12; $P < 0.05$), *twist* (fold change compared with control = 3.77; $P < 0.05$) and *tgf-β3* (fold change compared with control = 3.63; $P < 0.05$). Other genes studied (Table 2) were not significantly modulated.

Effect of monoclonal antibody to the peptide backbone of CD24

MultiGene-12 Strips visualized on a 2% agarose gel by RT-PCR arrays for 30 cycles for CD24 peptide antibody



C

Plasmid-no-CD24 siRNA (pg)	Plasmid-no-CD24 siRNA β-actin (pg)	Gene of interest (GOI)	Plasmid-with-CD24 siRNA (pg)	Plasmid-with-CD24 siRNA β-actin (pg)	Gene of interest (GOI)	Fold change of GOI (paired t-test: $P<0.01$)	Silencing CD24 mRNA percentage
2571	2689	0.96	34	312	0.11	0.11	89%
954.77	2146.85	0.44	95.87	1216.24	0.07	0.17	83%
207	1204	0.17	178	5024.97	0.00035	0.02	98%

Fig. 2. Specific down-regulation of mRNA for CD24 in H413 clone-1 cells. (A) The presence of the Neo resistance (*NeoR*) gene in each collected RNA sample was confirmed by RT-PCR. (B) Agarose gel showing CD24 mRNA silencing with two different inserts compared to plasmid without CD24 siRNA insert by RT-PCR for 30 cycles. No detectable CD24 mRNA expression in cells transfected with plasmid containing insert-1 and weak expression in cells transfected with plasmid containing insert-2. Real-time quantitative RT-PCR analysis confirmed the suppression of CD24 mRNA levels (panel C). The silencing of CD24 mRNA was between 83% and 98% in triplicate experiments.

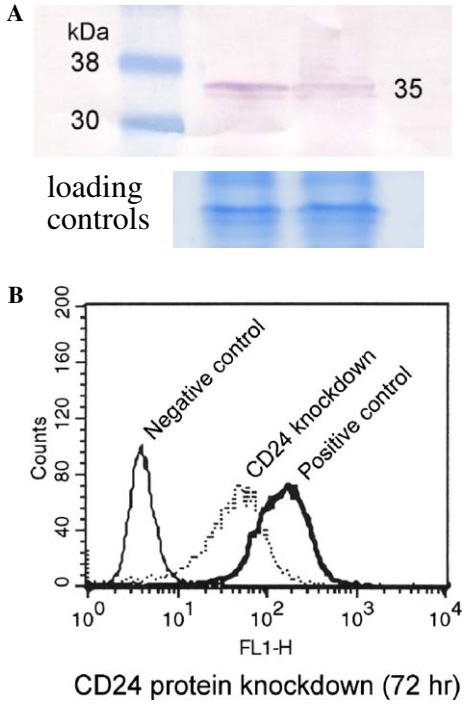


Fig. 3. Impact of siRNA on expression of CD24 protein in H413 clone-1 cells. (A) Western blot: lane 1 shows expression of CD24 in cells transfected with plasmid without inserts and lane 2 shows reduced CD24 expression in cells transfected with CD24 siRNA. (B) Flow cytometric analysis showing knock-down of CD24 protein (80% population) at 3 days after transfection.

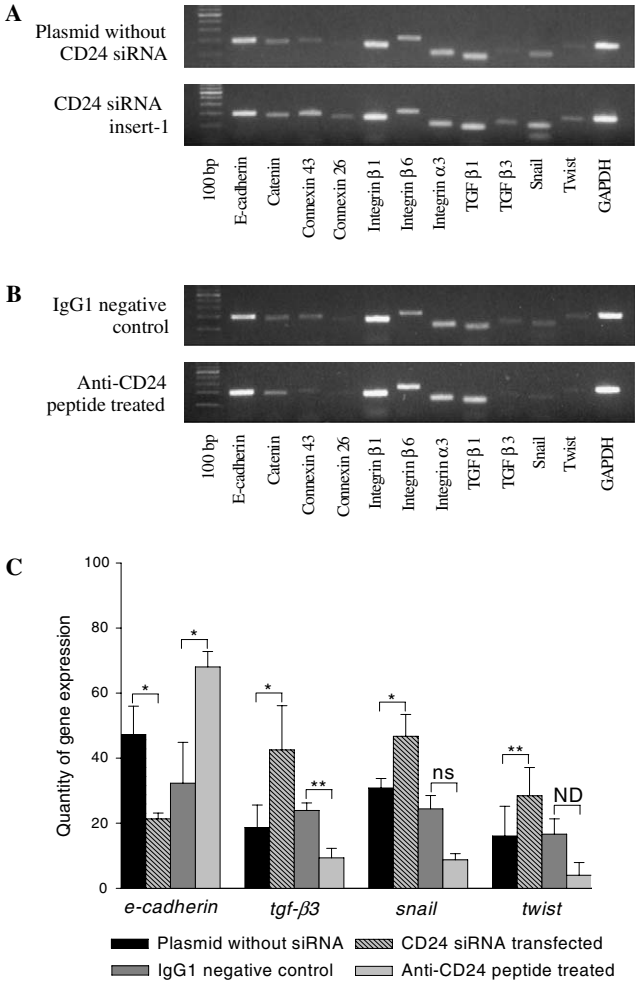


Fig. 4. Patterns of gene expression in H413 clone-1 cells related to manipulation of CD24. (A) siRNA/CD24. Representative analysis using MultiGene-12 Strips visualized on a 2% agarose gel following RT-PCR for 30 cycles. (B) Gene expression profiles in H413 clone-1 cells treated with monoclonal antibody to CD24 peptide compared to isotype control antibody. Representative analysis using gene Strips and visualized on a 2% agarose gel following RT-PCR arrays for 30 cycles. (C) Bar graph showing combined data for siRNA and anti-CD24 antibody studies. The values for each experiment were normalized against the expression of GAPDH (see Data analysis) and compared with the control sample. Columns depict mean values for three separate experiments together with s.e.m. Vertical axis indicates gene expression levels relative to the control GAPDH expression assigned a value of 100. *Refers to $P < 0.05$; **$P < 0.01$. ns = not significant. ND = not determined, but determined by real-time TaqMan RT-PCR (see Results).

treated H413 clone-1 cell showed changes in gene expression compared to the same isotype IgG1 antibody as a negative control (Fig. 4B). The quantitative data for experiments using triplicate assays when analyzed showed significantly increased E-cadherin mRNA expression ($P < 0.05$) and down-regulation of *tgf-β3* ($P < 0.01$) by paired *t*-test (Fig. 4C). No significant changes for *snail* expression were found, while accurate levels could not be determined for *twist* because of a weak signal. Real-time quantitative RT-PCR analysis confirmed that antibody to CD24 induced up-regulation of *e-cadherin* (fold change compared with control = 15.83; $P < 0.05$) and down-regulation of *tgf-β3* (0.56-fold of control; $P < 0.05$). No significant change was detected for *snail* but up-regulation for *twist* (fold change compared to control = 3.1; $P < 0.05$ [six experiments]) was found. There were no significant changes in the expression of the other genes studied.

Discussion

A central reference for strategies employed in gene silencing is the Editorial in Nature Cell Biology [22]. We used a plasmid without insert control as mis-matched sequence controls gave the spurious result of effective modulation of CD24 peptide levels without demonstrable effect on mRNA levels of the targeted gene. In preliminary investigation, siRNA plasmid vectors [19] were demonstrated to yield consistent knock-down compared with inconsistent results for synthetic siRNAs for CD24. A major concern is the induction of a so-called interferon response by short double stranded RNA sequences [20] that was excluded as a possibility by the analysis of OAS1 mRNA levels [18]. A further recommendation for evaluating specificity was the testing of multiple siRNA inserts. In this study insert-1 was the most effective producing a mean suppression of 90% (range from 83% to 98%), insert-2 was demonstrably less effective (Fig. 2B), while inserts-3 and -4 had minimal impact on CD24 mRNA levels, providing effective controls for the specificity of siRNA knock-down of CD24 mRNA. Accordingly, specific knock-down of CD24 mRNA by

siRNA was achieved and validated by real-time RT-PCR, with reduced expression of CD24 protein confirmed by analytical flow cytometry.

Extensive literature supports the critical role of E-cadherin in the maintenance of epithelial integrity by the functionality of Ca^{++} bridged homophilic interaction of the extracellular domains of E-cadherin focused within adherens junctions on opposing cells [23]. The junctional epithelium adherent to the tooth surface is highly permeable [1], a feature preserved in the reactive lining epithelium of periodontitis [4]. This is considered to facilitate leukocyte migration but also to allow egress of bacteria and bacterial products from the proximal microbial biofilm into the tissues [1]. Regulation of E-cadherin expression may be significant in maintaining this functionality. While multiple gene products and regulatory events impact on the association of the intracellular domain of E-cadherin through to F-actin [23], the expressed protein has a short half-life of 5–10 h and therefore alteration of mRNA levels for this product rapidly change E-cadherin levels with major effects on epithelial structure [24]. In the culture model used, H413 oral epithelial cells responded to specific suppression of CD24 mRNA and the associated reduction of surface CD24 protein by down-regulating E-cadherin expression. Expression of other epithelial genes monitored including β catenin, connexins and integrins was not affected indicating a degree of specificity in targeting the e-cadheringene. Ligation of CD24 on un-manipulated H413 cells using monoclonal antibodies recognizing either peptide or carbohydrate antigens (data not shown) resulted in strong stimulation of E-cadherin mRNA expression but without change in CD24 mRNA (data not shown) or of mRNA's for the other epithelial structural genes studied. The data are interpreted as indicating that by ligation with other presently unknown moieties, signal transduction through CD24 provides important stimulation of e-cadheringene expression in un-manipulated cultured cells. Further, that challenge with antibody to CD24 specifically enhanced this effect. Auto-reactive antibodies with specificity for CD24 could have similar functions.

In siRNA-treated cells suppression of E-cadherin mRNA was associated with enhanced expression of *snail*, *twist*, and *tgf-β3*. The transcription factor Snail is a well-documented inhibitor of expression *e-cadherin* through a defined mechanism involving binding to a control box in close proximity to the promoter for *e-cadherin* [25]. Therefore the increase in expression of *snail* is a plausible mechanism for down-regulation of *e-cadherin* expression. Twist is considered to mediate epithelial-mesenchymal transitions in development and in neoplastic cells by inducing the expression of N-cadherin [26,27]. Antibody-mediated enhancement of mRNA for E-cadherin was uncoupled from both *snail* and *twist*. Thus there was no significant change for *snail* expression and expression of *twist* although at low levels by analysis of gene arrays, was shown by real-time RT-PCR to be increased, a finding confirmed over six experiments. TGF-β3 was, however, down-regulated in response to specific anti-CD24 challenge. This isoform of the transforming growth factor family was shown to be more potent than TGF-β1 or TGF-β2 in suppressing epithelial mitosis in the context of wound healing [28]. TGF-β3 is not normally expressed by epithelial tissues [6] and it is the TGF-β1 isoform that has been implicated in epithelial-mesenchymal transition related to the late stage of neoplastic progression [29]. In this context the pattern of expression by H413 clone-1 cells models that observed for the reactive epithelia of periodontitis [5]. TGF-β3 has been implicated in epithelial-mesenchymal transition in the developmental sequence of palatogenesis and it is noteworthy that it is expressed by the targeted epithelial cells in exact spatial and temporal context [30]. Complementary data sets from siRNA and antibody studies indicate that TGF-β3 is a plausible regulatory agent for CD24-mediated stimulation of E-cadherin expression.

At first glance the effect of CD24 in maintaining epithelial tissue integrity is paradoxical in light of increasing evidence that high level expression of this protein is an indicator of poor prognosis for a number of neoplastic diseases [31]. This is considered to relate to the capacity of CD24 to mediate haematogenous metastasis by interaction with P-selectin [32] rather than any known perturbation of epithelial structure *per se*. The findings of this study indicate in contrast, an interesting role for this receptor in the maintenance of epithelial integrity.

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