The transcription factors Slug and Snail act as repressors of Claudin-1 expression in epithelial cells[1]

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Claudin-1 is an integral membrane protein component of tight junctions. The Snail family of transcription factors are repressors that play a central role in the epithelial–mesenchymal transition, a process that occurs during cancer progression. Snail and Slug members are direct repressors of *E-cadherin* and act by binding to the specific E-boxes of its proximal promoter. In the present study, we demonstrate that overexpression of *Slug* or *Snail* causes a decrease in transepithelial electrical resistance. Overexpression of *Slug* and *Snail* in MDCK (Madin–Darby canine kidney) cells down-regulated Claudin-1 at protein and mRNA levels. In addition, Snail and Slug are able to effectively repress human *Claudin-1*-driven reporter gene constructs containing the wild-type promoter sequence, but not those with mutations in two proximal E-box elements. We also demonstrate by band-shift assay that Snail and Slug bind to the E-box motifs present in the human *Claudin-1* promoter. Moreover, an inverse correlation in the levels of *Claudin-1* and *Slug* transcripts were observed in breast cancer cell lines. E-box elements in the *Claudin-1* promoter were found to play a critical negative regulatory role in breast cancer cell lines that expressed low levels of *Claudin-1* transcript. Significantly, in invasive human breast tumours, high levels of *Snail* and *Slug* correlated with low levels of *Claudin-1* expression. Taken together, these results support the hypothesis that *Claudin-1* is a direct downstream target gene of Snail family factors in epithelial cells.

Key words: Claudin-1, E-cadherin, Slug, Snail, tight junction, tumour.

INTRODUCTION

TJs (tight junctions) constitute continuous circumferential seals around cells and serve as the primary barrier, preventing solutes and water from passing freely through the paracellular pathway [1]. One of the most important functions of TJs is to maintain the differentiated state of epithelial cells. An absence of TJs, or defective TJ formation, has been associated with the development of neoplastic phenotypes in normal epithelial cells [2–4]. Among the proteins present in TJs, Claudin-1 is a major constituent [5,6]. Several lines of evidence suggest that Claudin-1 is directly involved in the barrier and fence functions of TJs [1,7].

Human Claudin-1 was identified by differential display, comparing the mRNA of proliferating, early passage, and normal HMECs (human mammary epithelial cells) with the mRNA of normal senescent HMECs [8]. cDNA levels proved low in proliferating HMECs compared with the high expression detected in senescent HMECs; interestingly, the absence or significantly reduced expression of *Claudin-1* has been observed in several established breast cancer cell lines [8]. Comparison of the expression profile of *Claudin-1* in non-malignant cells with that in tumour-derived cells reveals this gene to be a key player in tumorigenesis, primarily by acting as a suppressor of mammary epithelial proliferation [8]. Analysis of the coding region of *Claudin-1* in sporadic tumour cells and hereditary breast cancer patients did not reveal a clear relationship between alterations in *Claudin-1* and its expression pattern. Furthermore, mutational analysis of the *Claudin-1* gene and its putative promoter in breast cancer cell lines did not indicate any apparent modification [9].

Snail family members encode zinc-finger transcription factors that are essential for mesoderm formation in several organisms, from flies to mammals [10]. More recently, this role in promoting cell movement has been elucidated further to include more generalized phenomena such as EMT (epithelial–mesenchymal transition), a process that occurs at defined stages of embryonic development and during cancer progression [11–13]. EMT involves the conversion of an epithelial cell into a mesenchymal cell, one characterized by a more motile, invasive and aggressive phenotype. These changes allow some tumour cells to migrate through the extracellular matrix and colonize lymph/blood vessels in the first steps of the metastatic process. In the last few years, great advances have been made in understanding the EMT process and several critical molecules have been identified. Snail and Slug have now been firmly established as repressors of *E-cadherin*, one of the key molecules in the EMT process, both in early development and in cancer progression [11,12]. However, additional target genes are most likely required to explain the role of Snail in cell migration and cancer development, such as the recently identified *mucin-1*, *collagen IIa1* or *MMP-2* (matrix metalloproteinase-2) genes [14–16].

In the present study, we show that overexpression of *Slug* or *Snail* in MDCK (Madin–Darby canine kidney) cells led to

Abbreviations used: c, canine; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; EMT, epithelial–mesenchymal transition; ERK, extracellular-signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GST, glutathione S-transferase; HMEC, human mammary epithelial cell; m, mouse; MDCK, Madin–Darby canine kidney; pAb, polyclonal antibody; TEER, transepithelial electrical resistance; TJ, tight junction; ZO-1, zonula occludens-1.

[1] This paper is dedicated to the memory of our friend and colleague, Senén Vilaró.

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The nucleotide sequence data reported for *Canis familiaris* partial mRNA for putative claudin-1 protein (*cldn1* gene) has been submitted to DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AJ628857.

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a dramatic down-regulation of Claudin-1 protein levels and a significant reduction of *Claudin-1* mRNA. The E-boxes in human *Claudin-1* promoter are responsible for the Slug- and Snail-induced repression of its promoter activity, exerting both a critical and negative regulatory role in breast cancer cell lines that express low levels of the *Claudin*-1 transcript. Significantly, in invasive human breast tumours, high levels of *Snail* and *Slug* have been correlated with low levels of *Claudin-1* expression. These observations suggest that the Snail family of transcription factors are strong candidates for mediating the repression of *Claudin-1* expression in epithelial cells.

EXPERIMENTAL

Antibodies, recombinant proteins and cells

Reagents were purchased from Sigma, unless stated otherwise. Rabbit pAbs (polyclonal antibodies) were used to detect Claudin-1 and ZO-1 (zonula occludens-1) (Zymed). E-cadherin monoclonal antibody was purchased from Transduction Laboratories. Polyclonal antibodies against Snail (E-18) and Slug (H-140) were purchased from Santa Cruz Laboratories and polyclonal antibodies against GFP (green fluorescent protein) were from Clontech. MDCK cells stably transfected with *Snail*, *Slug* or pcDNA3 (control) have been described previously [11,17]. Primary fibroblast and the human breast cancer cell lines MDA-MB231, MDA-MB435, MDA-MB468, MCF-7 and T47D were obtained from the Cell Line Collection of Barcelona University (EucellBank). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) foetal calf serum. All experiments were performed with at least two clones. Transwell polycarbonate membrane inserts were purchased from Costar. Radioactive products were obtained from Amersham Biosciences. GST (glutathione S-transferase)–Snail and GST–Slug were produced as described previously [12,17].

Measurement of TEER (transepithelial electrical resistance)

For TEER measurements, 1×10^5 transfected cells were plated on Transwell polycarbonate membrane inserts with a pore size of 0.4 μm and an area of 1.1 cm^2. A Millicell-ERS volt–ohmmeter (Millipore) was used to determine the TEER value. The Millicell-ERS system was used in accordance with the manufacturer's instruction. Calculations for ohm $\times$ cm^2 were made by subtracting values of blank inserts from all samples and multiplying by the area of the monolayer. TEER values were determined after 48 h in culture in DMEM supplemented with 10 % (v/v) foetal calf serum. TEER experiments were performed in triplicate for each transfected cell.

Immunofluorescence and Western blot analysis

Cells grown on coverslips were rinsed with PBS, fixed with 3 % (w/v) paraformaldehyde for 20 min at room temperature (22 °C), and were permeabilized using 0.5 % (v/v) Triton X-100. Monolayers were processed for indirect immunofluorescence with pAbs against Claudin-1 (1:100), ZO-1 (1:100) or E-cadherin (1:100), incubated with FITC-conjugated anti-rabbit or anti-mouse IgGs (Dako) and were analysed using confocal microscopy [18]. For Western blotting, 20 μg of proteins (whole extracts) were separated by SDS/7.5 % PAGE and transferred on to nitrocellulose membranes (Schleicher & Schuell), and protein expression was analysed as described previously [18].

RT (reverse transcription)–PCR analysis

Total RNA was isolated from the different transfected cell lines using an RNA purification kit (Invitrogen). Mouse and canine PCR products were obtained after 30–35 cycles of amplification with an annealing temperature of 60–65 °C using a SuperScript One-Step RT–PCR kit (Invitrogen). Primer sequences were as follows. For canine *Claudin-1* (c*Claudin-1*): forward, 5′-CGGT-TCTGCGTCTCAGTTC-3′, and reverse, 5′-GTTGCCCATGAC-TCGCTC-3′; the primer pair amplified a fragment of approx. 250 bp. Database searches with the putative c*Claudin-1* sequence revealed 96 % identity with human *Claudin-1*. For canine *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase): forward, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, and reverse, 5′-CATGTAGGCCATGAGGTCCACCAC-3′; the primer pair amplified a fragment of approx. 900 bp. For canine *Snail* (c*Snail*): forward, 5′-CCCAAGCCCAGCCGATGAG-3′, and reverse, 5′-CTTGGCCACGGAGAGCCC-3′ (amplified a fragment of approx. 200 bp). For mouse *Snail* (m*Snail*): forward, 5′-GGCGGA-TCCACCATGCCGCGCTCCTTCCTGGTC-3′, and reverse, 5′-CCGGATATCCGCGAGGGCCTCCGGAGCA-3′ (amplified a fragment of approx. 800 bp). For canine *Slug* (c*Slug*): forward, 5′-AGTGATTATTTCCCCATATCTCTATGA-3′, and reverse, 5′-GTAGTCTTTCCTCTTCATCACTAATGG-3′ (amplified a fragment of approx. 260 bp). For mouse *Slug* (m*Slug*): forward, 5′-CGCGAATTCCCGCCCGCAGCCACC-3′, and reverse, 5′-ACT-CTCGAGCTAGTGTCAATGGGCGAC-3′ (amplified a fragment of approx. 850 bp).

Quantitative real-time PCR

Total RNA (1 μg) from each breast cancer cell line was reverse-transcribed in a final volume of 50 μl using Taqman reverse transcription reagents (Applied Biosystems). Real-time PCR on 20 ng of cDNA was performed for each of the following genes using assays-on-demand from Applied Biosystems: human *Claudin-1* (hs00221623), *Snail* (hs00195591), *Slug* (hs00161904) and *GAPDH* (hs99999905). All PCRs were performed using an ABI Prism 7700 sequence detection system. For any sample, the expression levels of *Claudin-1*, *Snail* and *Slug* were normalized to the housekeeping gene *GAPDH*. Relative mRNA expression levels were determined using the comparative threshold cycle method [19]. The graphics represent $[2^{-CT\,(target)}/2^{-CT\,(housekeeping)}]$ cell line/$[2^{-CT\,(target)}/2^{-CT\,(housekeeping)}]$ fibroblast cells.

Isolation of promoter fragments, mutagenesis and reporter assays

A human *Claudin-1* (-748 to $+252$) promoter fragment was amplified by PCR from genomic DNA of Caco2 cells and cloned into the pGL3 vector (Promega) upstream of firefly luciferase. PCR products were obtained after 40 cycles of amplification with an annealing temperature of 65 °C. Primer sequences were as follows: forward, 5′-GGAAACTACAGTCCCAGCGA-3′, and reverse, 5′-GATGTTGTCGCCGGCATA-3′ (amplified a fragment of 1384 bp). The fragment was digested using NheI/PvuII and cloned into NheI and SmaI sites of pGL3 vector (large promoter). The short construct (-82 to $+236$) was made by PCR-based site-directed mutagenesis. The PCR products were gel-purified and cloned using standard procedures. These reporter constructs (300 ng) were transfected into cells cultured in 24-well plates, in the absence or presence of the indicated amounts of pcDNA3 (control), pcDNA3-Snail or pcDNA3-Slug vectors; the total amount of transfected DNA was normalized with empty pcDNA3 plasmid. At 24 h after transfection, firefly luciferase (Luc) activity was measured using the Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions. In all experiments, luciferase activity was normalized by taking into account the co-transfection efficiency of a GFP vector. The percentages of GFP-positive cells were analysed in a FACScalibur microflow cytometer (Becton Dickinson).

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To mutate each E-box sequence in the *Claudin-1* promoter, a QuikChange Site-Directed Mutagenesis kit (Stratagene) was used. The core sequence, 5′-CA(G/C)(G/C)TG-3′, was mutated to 5′-TG(G/C)(G/C)TA-3′. The insert vector, deletions and each combination of mutations were confirmed by sequencing.

EMSA (electrophoretic mobility-shift assay)

The double-stranded oligonucleotides used as probes for gel retardation corresponded to the following sequence in the *Claudin-1* human promoter (+165 to +201): 5′-GTTGCC**CACCTG**CA-AACTCTCCGCCTTCTG**CACCTG**CACCCC-3′ (E-boxes are indicated in bold). The probe was end-labelled with [α-^{32}P]CTP using Klenow enzyme. Sequences of oligonucleotides used as competitors were as follows: E1 box (+153 to +192), 5′-TC-GGGAGTCCGGGTTGCC**CACCTG**CAAACTCTCCGCCT-TC-3′; E2 box (+176 to +215), 5′-GCAAACTCTCCGCCTTC-TG**CACCTG**CCACCCCTGAGCCAG-3′. In mutated probes, the core sequence 5′-**CACCTG**-3′ of E1 and E2 boxes was mutated to 5′-**TGCCTG**-3′.

Gel-retardation assays were performed as described previously [12]. Briefly, 10 ng of recombinant proteins were incubated for 30 min with 2.5 ng of radiolabelled oligonucleotides. Binding buffer contained 20 mM Hepes, pH 7.6, 150 mM KCl, 3 mM $MgCl_2$, 4 % Ficoll, 0.1 % Nonidet P40, 1 mM dithiothreitol, 1.5 μM $ZnCl_2$ and 0.5 mg/ml BSA. The reactions were supplemented as indicated in Figure 5. For the competition experiments, unlabelled oligonucleotides were added 10 min before the labelled ones. To detect band supershifts, antibodies (anti-Snail, anti-Slug or non-immune serum) were added after this step for 15 min at room temperature. Complexes were resolved on 4 % acrylamide gels (19:1 acrylamide/bisacrylamide) prepared in 22 mM Tris/borate.

Microarray analysis

Using microarray analysis and samples from the fresh-frozen tissue bank of the Netherlands Cancer Institute, primary invasive breast carcinomas from a series of 295 consecutive women with breast cancer were studied by van de Vijver et al. [20]. Briefly, total RNA from each tumour was isolated and used to generate cRNA, which was then labelled and hybridized into microarrays containing approx. 25 000 human genes. Fluorescence intensities of scanned images were quantified and normalized. The ratio was calculated with respect to the intensity of a reference pool made up of equal amounts of cRNA from all tumours [20]. From this dataset, we selected the entries containing the expression vectors for our genes of interest: *Snail*, *Slug* and *Claudin-1*. In order to identify trends in the expression levels of these genes, complete linkage hierarchical clustering was conducted on the normalized median-centred expression values using Euclidian distance [21].

RESULTS

Overexpression of *Slug* or *Snail* induces a disruption of TJs

To determine whether Slug and/or Snail may contribute to the increased permeability of TJs during EMT, we measured the TEER of *Slug*/*Snail*-transfected MDCK cells (Figure 1). *Slug*- and *Snail*-expressing clones exhibited a complete abolition of TEER values, independently of the number of days in culture (results not shown). The reduction in TEER values proved to be unrelated to the levels of *Snail* and *Slug* expression.

Overexpression of *Slug* or *Snail* modifies *Claudin-1* expression

Using immunofluorescence microscopy, we examined Claudin-1 expression in MDCK (control) and MDCK clones derived after stable transfection with murine *Slug* or *Snail* [11,17]. As shown in Figure 2(A), control cells exhibited a regular linear labelling of Claudin-1, E-cadherin and ZO-1 at sites of cellular contact. However, both *Slug*- and *Snail*-transfected cells presented almost undetectable immunoreactivity for Claudin-1 and E-cadherin. The reduction in the labelling resulted from a dramatic decrease in protein levels of Claudin-1 and E-cadherin (Figure 2B). Consistent with previous observations [22], ZO-1 staining was redistributed from TJs in control MDCK cells to the cytoplasm in *Snail*- and *Slug*-transfectants, while protein levels remained unchanged in both cell types (Figures 2A and 2B). To clarify the mechanism underlying Claudin-1 down-regulation, we performed semi-quantitative RT–PCR to detect the expression of c*Claudin-1* mRNA. In control cells, *Claudin-1* transcripts were abundantly expressed, while, in *Slug*- and *Snail*-transfected cells, they were down-regulated (Figure 2C). To investigate further the potential influence of *Snail* and *Slug* levels in the regulation of TJs, protein Western blot and immunofluorescence analyses of Claudin-1, Occludin and ZO-1 were conducted in different MDCK-*Snail* and -*Slug* clones. As controls, we also included E-cadherin and β-catenin, members of adherens junctions (Figure 2E). Interestingly, while we observed a dose-dependent inhibition of Claudin-1, Occludin and E-cadherin expression, ZO-1 and β-catenin levels remained almost unchanged. The subcellular distribution of TJ proteins changed from cellular contact in control MDCK cells to the cytoplasm in *Snail*- and *Slug*-transfectants cells (Supplementary Figure 1S at http://www.BiochemJ.org/bj/394/bj3940449add.htm). In addition, the reduction of *Claudin-1* transcription levels also occurred in a dose-dependent manner (Figure 2F).

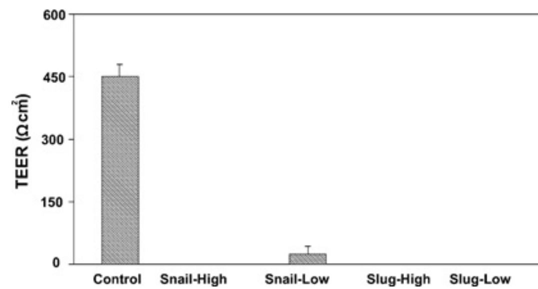

Figure 1 Snail and Slug induce a disruption of TJs in epithelial cells

Snail-, *Slug*- and pcDNA3- (control) stably transfected MDCK cells (1×10^5) were plated on Transwell polycarbonate membrane inserts with an area of 1.1 cm^2. TEER values were determined after 48 h in culture in DMEM supplemented with 10 % (v/v) foetal calf serum. *Snail*- and *Slug*-MDCK overexpressing clones (Snail-High, Snail-Low, Slug-High and Slug-Low) exhibited a complete abolition of TEER values when compared with control cells, independent of transcription factor expression levels. Results are means $\pm$ S.D. for three independent experiments, each performed in triplicate.

Slug and Snail function as direct repressors of the *Claudin-1* gene

We subsequently examined whether or not the *Claudin-1* gene is directly regulated by the Snail family members Slug and Snail (Figure 3). To this end, we isolated the promoter region of the human *Claudin-1* gene (−748 to +252) [9] and fused it to the luciferase reporter cDNA. Transient transfection assays in the prototypic epithelial cell line MDCK in the presence of pcDNA3-Slug or pcDNA3-Snail demonstrated that both Slug and Snail are able to repress *Claudin-1* promoter activity in a dose-dependent manner, although with apparent distinct efficiencies, inducing a 50 % and 70 % repression respectively, at 150 ng (Figures 3A and 3B). An additive effect was observed in the presence of 75 ng

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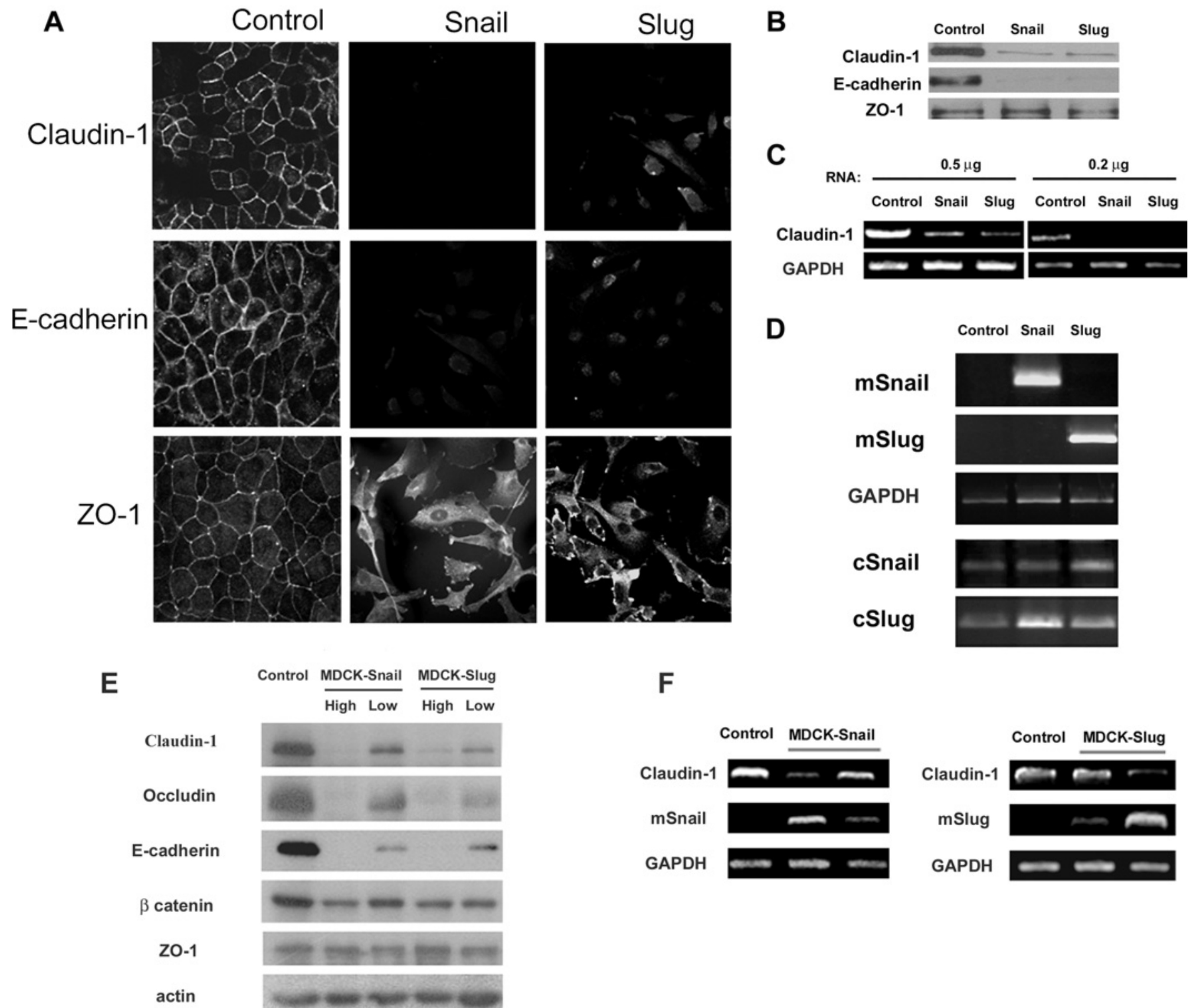

Figure 2 Overexpression of *Snail* and *Slug* are associated with a full repression of *Claudin-1* expression

(**A**) Control (pcDNA3), *Snail*- and *Slug*-transfected MDCK clones were analysed by immunofluorescence and confocal microscopy for the expression of *Claudin-1*, *E-cadherin* and *ZO-1*. In *Snail*- and *Slug*-transfected cells, Claudin-1 and E-cadherin became almost undetectable. (**B**) Western blot analysis of whole-cell extracts for the indicated protein was conducted. *Snail* and *Slug* transfection caused a reduction in the protein levels of Claudin-1. (**C**) The reduction in the protein levels of Claudin-1 was caused by a reduction in the transcript. The presence of c*Claudin-1* transcripts in control, *Slug*- and *Snail*-transfected clones was analysed by RT–PCR using different amounts of RNA. The expression of *GAPDH* was analysed in the same sample as a control. (**D**) RT–PCR for m*Snail*, m*Slug* c*Snail*, c*Slug* and *GAPDH* expression in control, *Snail* and *Slug* clones. (**E**) Expression patterns of TJ and adherens junction proteins in control and transfected cells with different levels of *Snail* and *Slug* expression. (**F**) RT–PCR for c*Claudin-1*, m*Snail*, m*Slug* and *GAPDH* expression in control and different *Snail* and *Slug* clones. Representative data from three independent experiments are shown.

of each transcription factor (Figure 3C). These findings indicated that the transcription promoter activity of human *Claudin-1* was regulated directly by both Slug and Snail factors. To account for the apparent discrepancies between the study published by Ohkubo and Ozawa [23] and our own findings, we obtained a short promoter of human *Claudin-1* gene (−82 to +236) [23]. Both promoters were tested in MDCK cells. Consistent with our RT–PCR analysis, the shorter promoter of *Claudin-1* was repressed by Snail and Slug in a manner similar to that of the large promoter (Figure 3D).

To clarify further the molecular mechanism underlying the Slug- and Snail-induced repression of *Claudin-1* transcription, we examined the human *Claudin-1* promoter in detail and found two E-box motifs (Figures 4A and 4B) conforming to the Snail-binding E-boxes (CACCTG) at +171 and +189. Interestingly, the two E-boxes and adjacent sequences were fully conserved between human and canine *Claudin-1* genes (Figure 4B). We generated a series of reporter constructs that carried individual or double mutations in the E-boxes of the human *Claudin-1* promoter. Tellingly, constructs with a single mutated E-box (M1 or M2) became less sensitive to Slug and Snail repression. In addition, an additive effect was observed when both E-boxes were mutated, the double-mutated construct (M1-2) becoming insensitive to Slug and Snail repression, thereby suggesting that the two proximal E-boxes in the *Claudin-1* promoter are responsible for Slug- and Snail-induced repression (Figure 4C).

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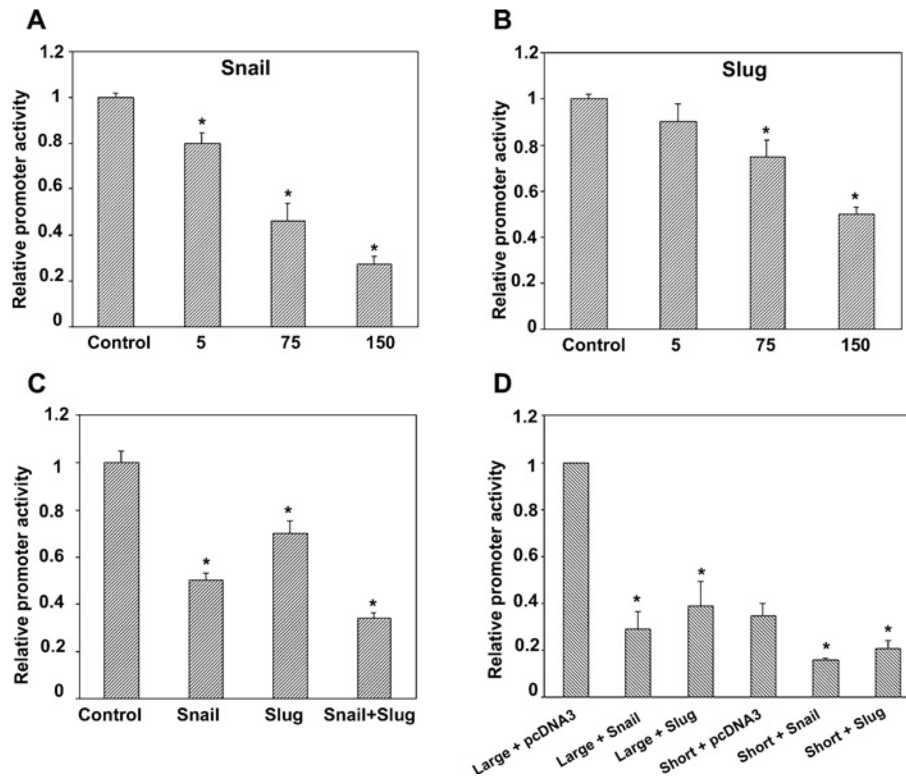

Figure 3 Snail- and Slug-induced repression of *Claudin-1* promoter activity

Luciferase reporter constructs carrying the human *Claudin-1* promoter (-748 to $+252$) were transfected in MDCK cells (300 ng) with empty vector (pcDNA3) (control), or together with *Snail* or *Slug*, or a combination of both factor expression constructs. (**A**, **B**, **C**) When *Snail* or *Slug* was co-expressed in MDCK cells, the activity of the *Claudin-1* reporter construct was repressed in a dose-dependent manner, an additive effect that was observed when both factors were co-transfected (**C**). (**D**) Luciferase reporter constructs carrying a large or short fragment of the human *Claudin-1* promoter (300 ng) were transfected in MDCK cells, together with (150 ng) *Snail* or *Slug* expression constructs, or with an empty vector (pcDNA3). A shorter fragment was also sensitive to Snail and Slug repression. Results are means $\pm$ S.D. for three independent experiments, each performed in quadruplicate. *, $P < 0.05$.

Snail and Slug bind directly to E-boxes in the *Claudin-1* promoter

We confirmed that Slug and Snail proteins interact directly with their putative binding sites in the *Claudin-1* promoter by using an EMSA (Figure 5). Recombinant mSlug and mSnail fused to GST, efficiently binding the proposed target sequences (boxes E1 and E2). GST–Snail formed three retarded complexes, while GST–Slug formed two complexes. Competitions were performed with a 200-fold excess of wild-type or mutant E1 and E2 cold probes. The specificity of each retarded complex was demonstrated by the fact that their formation was affected by unlabelled wild-type oligonucleotides, but not by unlabelled mutated oligonucleotides. Indeed, mutation of either of the two E-boxes was sufficient to block competition of the complexes generated by GST–Snail or GST–Slug with the labelled oligonucleotide, suggesting that both E-boxes are required for effective binding of the recombinant factors. Supershift experiments were performed by adding 200 ng of anti-Snail, anti-Slug or non-specific antibody. The presence of supershifted bands that appeared when incubating the complexes with an antibody directed against Snail or Slug, but not in the negative-control antibody, indicates that Snail and Slug are present in the complex.

Expression of *Snail*, *Slug* and *Claudin-1* in human breast cancer cell lines and in human breast tumour samples

To define the putative repressor role of Snail and Slug in *Claudin-1* expression in tumours of epithelial origin, real-time PCRs of these genes were conducted using a panel of epithelial breast cancer cell lines (Figure 6A). The low levels of *Claudin-1* transcripts in these cell lines inversely correlated with *Slug* expression (Spearman coefficient, $r = -0.64$; $P = 0.042$), while for *Claudin-1* and *Snail* the Spearman coefficient indicated an inverse correlation, though it did not reach statistical significance ($r = -0.15$; $P = 0.6$). The lowest levels of *Claudin-1* expression corresponded to the invasive metastatic cell lines, MDA-MB435 and MDA-MB-231, which also expressed the highest levels of the *Slug* transcript. However, the non-metastatic cells lines MCF-7 and T47D expressed the highest levels of *Claudin-1* and lower levels of *Slug*. To assess the putative role of Snail and Slug in the repression of *Claudin-1* in breast cancer cell lines, luciferase experiments were conducted with the wild-type or E-box-mutated constructs. A strong reduction in the wild-type promoter was observed in almost all of the cell lines tested. Inhibition was dependent on the E-box elements of the *Claudin-1* promoter in three of five breast cancer cell lines tested (Figure 6B). We then determined whether or not expression of *Snail* and *Slug* correlated with *Claudin-1* in clinical breast tumour samples. To this end, we analysed a microarray gene expression data set from 295 invasive human breast tumours produced by van de Vijver et al. [20]. Figures of Merit analysis applied to the sample category suggested the presence of four main clusters. Figure 6(C) represents a hierarchical clustering of the samples, where each row corresponds to a gene of interest and each column represents the relative level of gene expression in a given tumour sample. Red indicates a high level of mRNA expression in the tumour, compared with the reference mRNA level, and green indicates a

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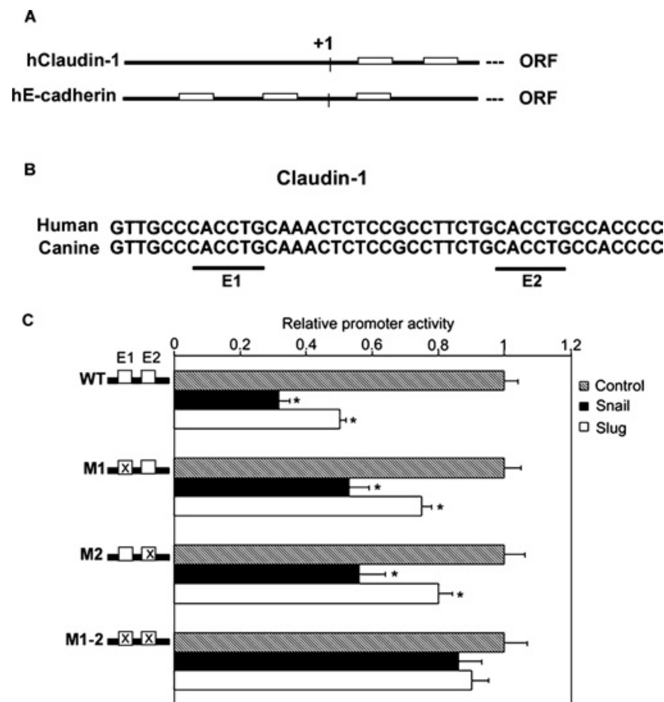

Figure 4 Impairment of Snail- and Slug-induced repression of *Claudin-1* promoter activity by mutations in the E-boxes

(**A**) Schematic representation of the E-boxes in the promoter region of human *Claudin-1* and *E-cadherin*: open box, E box; +1, putative transcription star point; ORF, open reading frame. (**B**) Comparison between the 5′-flanking region of human and canine *Claudin-1*. Alignment of the sequences revealed the presence of conserved E-boxes (underlined). (**C**) Mutational analyses. The core 5′-CA(G/C)(G/C)TG-3′ sequence of the E-boxes (E1, E2 and E1-2) was mutated to 5′-TG(G/C)(G/C)TG-3′, in various combinations (shadowed boxes). Luciferase reporter constructs carrying wild-type or mutated boxes were transfected in MDCK cells (300 ng), together with (150 ng) *Snail* or *Slug* expression constructs or the empty vector (pcDNA3). Luciferase activity in cells co-transfected with wild-type reporter constructs and the pcDNA3 empty vector was defined as 1. Results are means ± S.D. for three independent experiments, each performed in quadruplicate. *, $P < 0.05$.

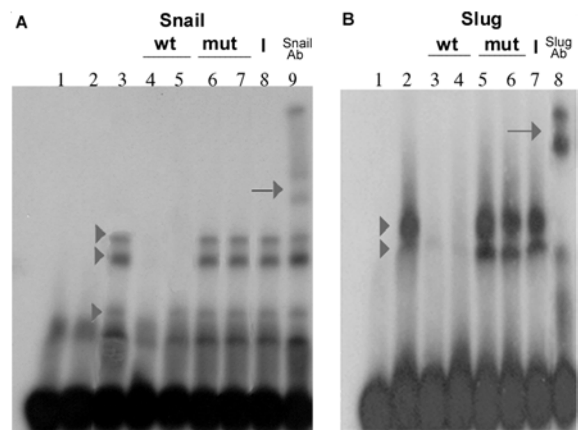

Figure 5 Direct binding of Snail and Slug to the E-boxes of the *Claudin-1* promoter

EMSA for the interaction of Snail and Slug with the E-box sequence. (**A**) Snail binding. Affinity-purified GST (lane 2) or GST–Snail (lanes 3–9) (10 ng) was incubated with double-stranded ^{32}P-labelled oligonucleotides (2.5 ng) (+165 to +201) corresponding to the sequence containing two E-boxes of the *Claudin-1* promoter; in lane 1, no protein was added. GST–Snail (arrowheads in lane 3), but not GST (lane 2), formed DNA complexes. Competitions were performed with a 200-fold excess of E1 (+153 to +192) and E2 (+176 to +215) wild-type probes (unlabelled oligonucleotides) (lanes 4 and 5), or mutant E1 and E2 boxes (lanes 6 and 7). Complex formation was affected by unlabelled wild-type E1 and E2 boxes, but not by unlabelled mutated boxes. Supershift experiments were performed by adding 200 ng of anti-Snail (lane 9) or non-specific (lane 8) antibody. Arrows in lane 9 indicate the supershifted retarded complexes. (**B**) Slug binding. Affinity-purified GST–Slug (lanes 2–8) (10 ng) was incubated with double-stranded ^{32}P-labelled oligonucleotides (2.5 ng), containing E1 and E2 boxes (+165 to +201); in lane 1, no protein was added. GST–Slug formed two DNA complexes (arrowheads in lane 2). Competitions were performed with a 200-fold excess of wild-type E1 and E2 (lanes 3 and 4), or mutant E1 and E2 boxes (lanes 5 and 6). Results were very similar to those shown in (**A**). Supershift experiments were analysed by adding 200 ng of anti-Slug (lane 8) or non-specific (lane 7) antibody. Representative results from six independent experiments are shown. wt, wild-type; mut, mutant; Ab, antibody.

low level of expression. Blue triangles were used to delimit the behavioural clusters.

The first group of tumours was characterized by a high expression of *Snail* when compared with the rest of the samples. A clear negative correlation between the levels of *Slug* and *Snail* expression can be observed. Despite the small variation observed in the expression of *Snail*, maximal *Claudin-1* expression was attained in those samples with the lowest expression of both transcription factors (Figure 6C, Detail 1). Low levels of *Slug* and intermediate levels of *Snail* characterized the second group identified. Here again, *Claudin-1* expression inversely correlated with the expression of *Snail* and *Slug*, proving maximal when both factors were at lower levels (Figure 6C, Detail 2). The third cluster behaved like the previous two. This cluster comprised the samples with lower *Snail* and *Slug* expression within the whole dataset. Interestingly, *Claudin-1* levels were at their highest. The fourth group contained samples in which *Slug* expression was at higher levels. *Claudin-1* expression was again inversely proportional to the expression of the transcription factors. Interestingly, this cluster contained the samples where a concomitant high expression of the transcription factors was found, correlating with the lowest levels of *Claudin-1* of the whole data set.

Taken as a whole, hierarchical clustering highlights the elevated heterogeneity within the studied tumours in the expression of the transcription factors *Snail* and *Slug*. Both factors seem to inversely correlate with the expression of *Claudin-1*, as their relative contribution, while constant, proved highly dependent on the tumour 'type'. Extreme effects on *Claudin-1* expression were observed when both factors were at their maximal or minimal levels, with any other combination generating only intermediate effects.

DISCUSSION

Several lines of evidence support an association between abnormalities in TJs and neoplasia [3,9,24]. To begin with, alterations in the number, appearance and permeability of TJs have been demonstrated in various cancer types [3,9,24,25]. It has been hypothesized that abnormalities in TJ permeability disrupt the concentration of luminal growth factors, allowing them to cross the epithelium and bind to receptors on the basolateral surface or on other cell types, triggering cell proliferation [4,7]. The loss of expression of *Claudin-1* has been demonstrated in several mammary carcinoma cell lines [8]. Furthermore, analysis of primary breast tumours has revealed a similar loss of *Claudin-1* expression in contrast with the broad expression spectrum found in other epithelial tissues [9]. However, mutation analysis of the *Claudin-1* gene and its putative promoter has provided no further insight into the loss of *Claudin-1* expression in breast cancer cell lines [9]. In addition, analysis of *Claudin-1* expression not only revealed that the Ras/MEK [mitogen-activated protein kinase/ERK (extracellular-signal-regulated kinase) kinase]/ERK pathway is not involved in the dysregulated TJ formation observed in breast tumour cells, but also indicated that the elevated activity

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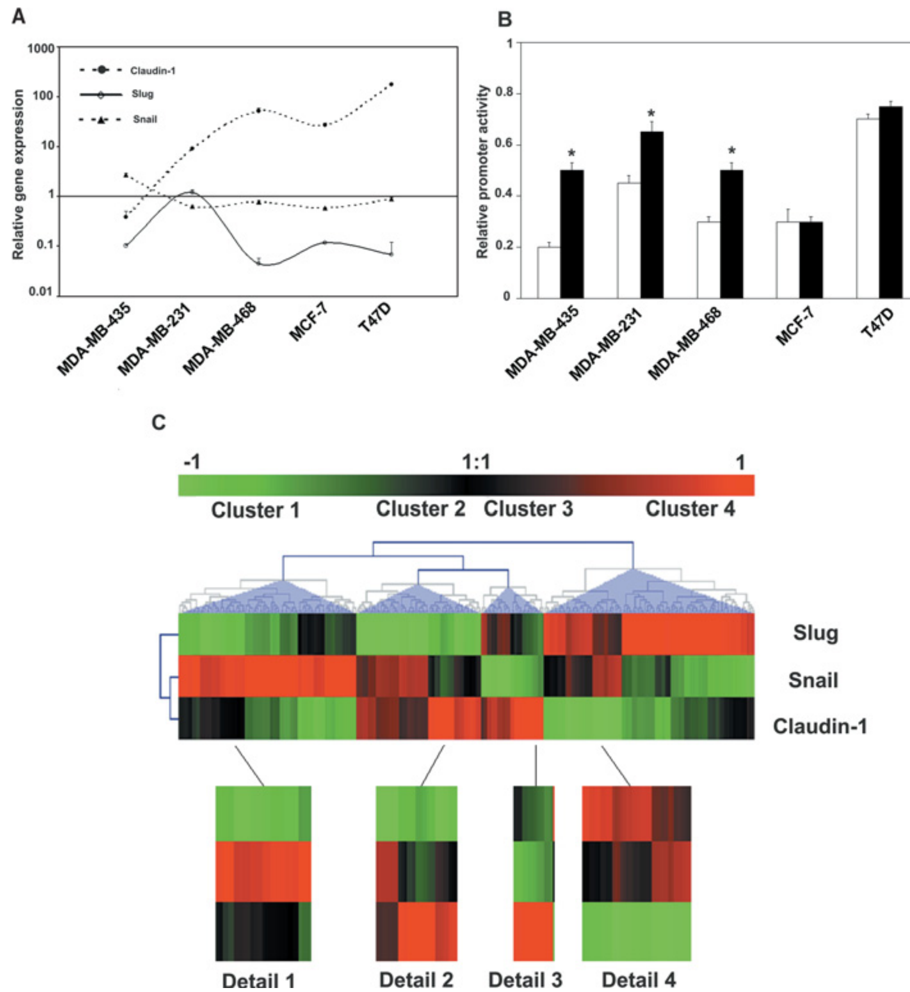

Figure 6 Expression of *Snail*, *Slug* and *Claudin-1* in breast cancer cell lines and in human breast tumour samples

(**A**) Total RNA from a panel of breast cancer cell lines was reverse-transcribed into cDNA. Results show the cell line/fibroblast ratio of normalized mRNA levels of *Claudin-1*, *Snail* and *Slug* using the comparative threshold cycle method. *Claudin-1* compared with *Slug*: Spearman $r = -0.64$; $P = 0.042$. *Claudin-1* compared with *Snail*: Spearman $r = -0.15$; $P = 0.6$. (**B**) Luciferase reporter constructs carrying wild-type or mutated boxes (300 ng) were transfected in a panel of breast cancer cell lines. Luciferase activity in MDCK co-transfected with wild-type reporter constructs was defined as 1. Results are means $\pm$ S.D. for three independent experiments. *, $P < 0.05$. (**C**) Gene cluster assay of 295 human primary invasive breast carcinoma samples using three genes: *Snail*, *Slug* and *Claudin-1*. For each gene, the ratio was calculated with respect to the intensity of a reference pool made up of equal amounts of cRNA from all tumours [20].

of Ras might not be of great importance for the disruption of such structures [26].

The Snail superfamily of zinc-finger transcription factors has emerged in recent years as an important regulator of EMT [11]. Snail and Slug have now been firmly established as repressors of *E-cadherin* in early development and in different murine and human carcinoma and melanoma cell lines and tumours [11,12,17,27]. Recently, it has also been shown that Snail is able to repress the expression of the TJ proteins Claudin-3, -4 and -7 and Occludin [22].

In the present study, we have extended the analyses on the regulation of TJ components to Slug, another member of the Snail family, providing evidence for the direct repressor effect on human *Claudin-1* gene expression. Our results suggest a new mechanism for Snail/Slug-induced TJ down-regulation in epithelial cells, and indicate that elevated levels of *Slug* and *Snail* may be important for the disruption of TJ structures in epithelial cells. We examined the behaviour of *Claudin-1* in *Slug*- and *Snail*-transfected MDCK cells and found that, similar to the Snail- and Slug-mediated down-regulation of *E-cadherin* transcription, Slug and Snail not only repress endogenous *Claudin-1* expression, but also induce a dramatic loss of TEER. The Slug- and Snail-induced repression of *Claudin-1* in MDCK cells is exerted at the transcriptional level and is dependent on the direct binding of Snail/Slug to proximal E-boxes as confirmed in *in vitro* and *in vivo* binding assays. In fact, the integrity of the proximal E-boxes of the human *Claudin-1* promoter is a requirement for the binding activity and repression effects of Snail and Slug, as confirmed by promoter activity and band-shift assays. In the proximal E-box cluster, Slug- and Snail-mediated repression of *Claudin-1* appears to occur primarily through the co-ordinated action of E1 and E2 boxes, although the requirement of additional regulatory elements to achieve this repression cannot be discarded at present.

The present results differ from a recent report [23] showing post-transcriptional down-regulation of Claudin-1 by Snail and the apparent absence of transcriptional regulation in MDCK cells. Such absences may be explained by differences in the levels of *Snail* expression in the transfected cell line used in both studies, as we demonstrated that there is an inverse dose-dependent correlation between *Snail* and *Slug* levels and the repression of the

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Claudin-1 gene. In addition, we demonstrated that a shorter promoter sequence is sensitive to the repression effects of Snail and Slug factors in MDCK cells. In the A431 cell line, the model used by Ohkubo and Ozawa [23] in the luciferase reporter assay, the basal activities of both constructs were dramatically reduced. In addition, although Snail and Slug were able to repress the large promoter, this repression proved smaller than in MDCK, as the small promoter was insensitive to repression (Supplementary Figure 2S at http://www.BiochemJ.org/bj/394/bj3940449add.htm). Real-time PCR analysis of the *Snail* and *Slug* genes in the A431 cell line demonstrated high levels of *Slug* expression and low levels of *Snail* expression (Supplementary Figure 3S at http://www.BiochemJ.org/bj/394/bj3940449add.htm). We speculated that in A431, a carcinoma cell line, the activities of both promoters were probably already repressed by Slug.

The data reported in the present paper support the idea that, when overexpressed, *Snail* and *Slug* can behave as potent repressors of *Claudin-1* in epithelial cells. While the regulatory mechanism underlying the formation and destruction of TJs have been examined extensively, promoter analyses of *Claudins* and *Occludin* are only just beginning. Recently, a repressor effect of Snail on *Claudin-3*, *-4* and *-7* and *Occludin* was reported [22]. The findings of the present study are the first to indicate that Slug can also control the expression of TJ proteins, in particular *Claudin-1*. As assessed by real-time PCR, an inverse correlation between the expression levels of *Claudin-1* and *Slug* factor was found in breast cancer cell lines. We observed that *Claudin-1* expression levels were lowest in the invasive and metastatic cell lines MDA-MB 231 and MDA-MB-435. Moreover, in these cell lines, the expression levels of *Slug* were higher than in non-metastatic tumour cell lines, such as MCF7 and T47D. Slug expression is increasingly being recognized as an alteration in mesenchymal tumours, suggesting that Slug, like Snail, may be a critical invasion factor [28]. The microarray gene expression data set from the 295 invasive human breast tumours reviewed in the present study demonstrated an inverse correlation between *Snail* and *Slug* expression and *Claudin-1*. We identified four groups of tumours, based on the expression levels of each protein analysed. Tellingly, when one of the transcription factors is expressed at very low levels, such as in groups 1 and 3, small changes in the expression levels of the other are able to induce a dramatic decrease in the *Claudin-1* levels. The highest levels of *Claudin-1* expression were observed in the second group, which corresponded to tumours with low levels of *Snail* and *Slug* expression. Interestingly, group 4 contained tumours with the lowest levels of *Claudin-1* and the highest levels of *Snail* and *Slug* expression.

Our data indicate that both Slug and Snail may serve as potential repressors of *Claudin-1* in epithelial tumour cells. This could explain the loss of *Claudin-1* expression in breast epithelial tumour cell lines and primary tumours. The existence of both Slug and Snail in non-tumorigenic epithelial cells suggests the presence of additional factors that contribute to their inhibitory effect in tumour epithelial cells. The specific role of each, or their potential co-operation, in specific cellular contexts and in different types of tumour cells remains to be fully elucidated.

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