Snail Up-regulates Proinflammatory Mediators and Inhibits Differentiation in Oral Keratinocytes

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Abstract

The transcriptional repressor Snail2 is overexpressed in head and neck squamous cell carcinomas (HNSCC) relative to nonmalignant head and neck mucosal epithelium, and in locally recurrent relative to nonrecurrent HNSCCs. We investigated the mechanisms by which Snails might contribute to the pathogenesis of HNSCCs using cell biological and molecular analyses. Oral keratinocytes that expressed Snails acquired an enhanced ability to attract monocytes and to invade a dense interstitial collagen matrix. They were also found to up-regulate production of proinflammatory cytokines and cyclooxygenase-2 (COX2), which have previously been shown to correlate with malignancy. Induction of nuclear factor-κB transcriptional activity by Snails was weak and not sufficient to account for the elevated levels of COX2, interleukin (IL)-6, IL8, or CXCL1. In addition, expression of Snails in oral keratinocytes impaired desquamation *in vitro* and strongly repressed expression of both ELF3 and matriptase-1, which play important roles in the terminal differentiation of keratinocytes. Reexpression of matriptase-1 in Snail-expressing cells partially rescued desquamation. This implicates Snails as contributing to malignancy both at the early stages, by impeding terminal differentiation, and at later stages, when invasion and inflammation are important. [Cancer Res 2008;68(12):4525–30]

Introduction

Head and neck cancer is the sixth most common cancer and has seen little improvement in mortality over several decades (1). The keratinocytes that give rise to head and neck squamous cell carcinoma (HNSCC) are characterized by impaired terminal differentiation, dysplasia, and invasion of extracellular matrices. HNSCCs are frequently associated with inflammation and proinflammatory cytokines (2). The transcription factor nuclear factor κB (NFκB) has been implicated in driving their expression, but the mechanisms underlying increased NFκB activity are not clear, and are complicated by the fact that some cytokines can themselves increase NFκB activity in target cells.

The Snail family of transcriptional repressors has been associated with several forms of carcinoma and has important roles in development and injury responses (3). In head and neck tissues, both malignancy and local recurrence following treatment have been associated with a gene expression signature that includes Snail2 (4). Snails can cause an epithelial-mesenchymal transition and invasion (3). In the present study, we investigated the biological effects of Snail expression in human oral keratinocytes to determine how increased levels might contribute to HNSCC malignancy. We found that Snails promote changes in keratinocyte behavior and gene expression that would facilitate progression to malignancy by altering their abilities to terminally differentiate, invade extracellular matrices, and recruit proinflammatory leukocytes.

Materials and Methods

Cell culture. Materials were obtained from Sigma-Aldrich, unless otherwise indicated. WSU-HN13 cells (5) were transfected using Polyfect (Qiagen) with a plasmid (pCEFL2) driving expression of TVA, the receptor for RCAS viruses, from the EF1α promoter and selected for resistance to 0.4 mg/mL G418. Clones were screened for TVA by fluorescence after immunostaining with antibodies and transduction with enhanced green fluorescent protein (EGFP)-Renilla luciferase (RLh) cloned into RCASYGWB, an RCAS proviral vector (6). One clone, HN13(TVA), was chosen for further studies. Genes were introduced into HN13(TVA) by transduction using RCASYGWB. Using this procedure, >90% cells were transduced. Open reading frames of human Snail1, Snail2, RelA, and matriptase-1 were amplified by PCR and cloned as COOH-terminal myc (Snails), Glu-Glu (RelA), or hemagglutinin (matriptase-1) epitope tag fusions into pCEFL2 and RCASYGWB. Snail1(delSNAG) lacks amino acids 2 to 7 of the SNAG domain, which is essential for repressor activity. Snail1-ERT2/pCEFL2 was constructed by cloning the Snail1 open reading frame 5′ to the ERT2 tamoxifen-inducible cassette (7) and transfected into MDCK and HN13 cells. Clones (MDCK/Snail1-ERT2 and HN13/Snail1-ERT2) were selected on their response to 4-hydroxytamoxifen (4OHT) morphologically and by repression of a mouse E-cadherin promoter luciferase reporter gene in transient transfections. Control cells (HN13/EGFP-ERT2) were stably transfected with a plasmid in which the enhanced green fluorescent protein (EGFP) open reading frame substituted for Snail1. HN13/Snail-ERT2 cells were induced with 4OHT continuously for 7 d before RNA isolation.

Transcription analysis. The human and mouse E-cadherin promoters were amplified by PCR from genomic DNA and cloned into pGL3r2.1basic (Promega). NFκB activity was measured using NFκB-Luc (Stratagene). Luciferase activities were measured with Dual-Glo reagents (Promega). Microarrays used Cy5- and Cy3-labeled cDNAs hybridized to a human GEM2 cDNA array as previously described (8). Specific mRNAs were quantitated by reverse transcription and real-time PCR with Superscript III and Platinum SYBR Green Supermix (Invitrogen) in a Rotorgene 6000 thermocycler/analyzer (Corbett Research), normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, and corrected for the amplification efficiencies of GAPDH and the gene of interest. Primer sequences are listed in Supplementary Table S1. Cytokines in media were quantitated by ELISA (ThermoScientific).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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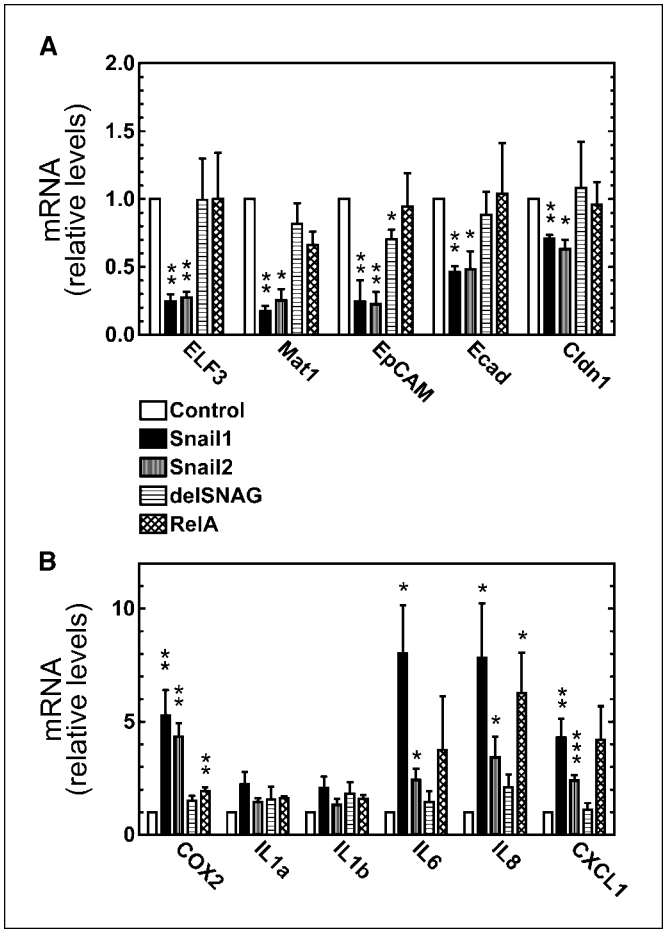

Figure 1. Regulation of Snail target genes in oral keratinocytes by Snails and RelA. HN13(TVA) keratinocytes were transduced with RCAS retroviral vectors expressing EGFP (*Control*), Snail1, Snail2, or Snail1-delSNAG (*delSNAG*), the latter lacking the SNAG domain required for repression, or RelA. Real-time PCR was used to determine the levels of mRNA of the Snail target genes. The Snail target signals were normalized to GAPDH and the levels relative to the control cells were calculated. The novel repressed Snail targets, ELF3, matriptase-1 (*Mat1*), and EpCAM, and the previously identified targets, E-cadherin (*Ecad*) and claudin-1 (*Cldn1*), are shown in *A*, whereas the up-regulated proinflammatory targets, COX2, IL1a, IL1b, IL6, IL8, and CXCL1, are shown in *B. Columns,* mean of three to nine experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significantly different from control cells (Student's *t* test).

Migration assays. Keratinocyte migration was assayed by using a blue pipette tip to "scratch" corridors of cells from a confluent monolayer, incubating for 16 h at 37C, fixing, and staining with Evans blue. The widths of 16 random places along the original scratch lines were measured using Canvas SE software (ACD Systems) and the "wound" closure was compared with the EGFP/RCASYGWB–transduced control cells. Human monocyte migration was measured by plating 10^5 THP-1 cells in fresh medium into the interior of an 8-μm porous polyethylene 24-well plate cell culture insert (Becton Dickinson) and filling the outer chamber with medium conditioned by HN13(TVA) cells or their Snail-expressing derivatives. After 20 h at 37°C, cells that had traversed the membrane were quantitated by incubation for 1 to 4 h in 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, lysing the cell pellet in DMSO, and reading the absorbance at 550 nm. Interleukin inhibitors were from R&D Systems.

Invasion assays. Invasion of collagen I matrix was measured by plating cells at a confluent density into 14-mm glass chamber dishes (MatTek) previously prepared by drying down FITC-labeled type I collagen that had been gelled at neutral pH (9). The resulting density of collagen is ~20 mg/mL, similar to that in a real tumor tissue. After 48 h, the cultures were fixed in 4% paraformaldehyde in PBS, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and imaged by confocal microscopy. Invasion was calculated from the positions of the nuclei of the leading cells relative to the top of the collagen matrix, as measured from Z-stacks.

Results

Activation of Snail1-ERT2 with 4OHT recapitulates Snail1 up-regulation in epithelial cells. Ginos and colleagues (4) identified Snail2 as part of a gene expression signature associated with malignancy and local recurrence of head and neck tumors. Further analysis shows a significant association of malignancy and local recurrence with Snail2 expression, independently of other genes (Supplementary Fig. S1). To identify Snail-regulated genes, Snail1 was expressed as a 4OHT-inducible COOH-terminal fusion protein (Supplementary Fig. S2*A*), enabling target genes to be identified by comparing 4OHT-induced and uninduced cells. To ensure that 4OHT induction of cells expressing Snail1-ERT2

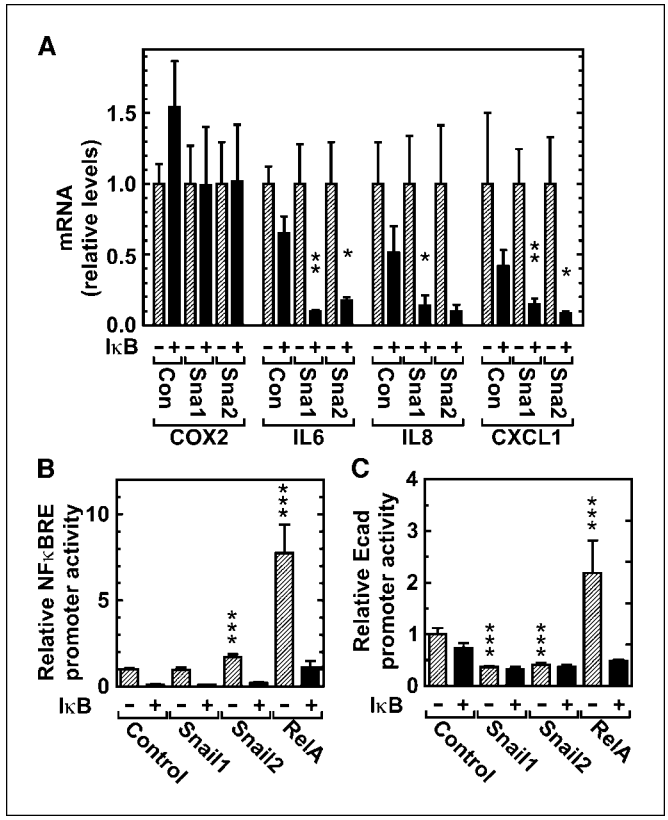

Figure 2. Transcriptional regulation by Snail in oral keratinocytes. *A,* HN13(TVA) keratinocytes were transduced with RCAS vectors expressing EGFP (*Con*; $n = 6$ independent experiments), Snail1 (*Sna1*; $n = 7$), or Snail2 (*Sna2*; $n = 6$), followed by EGFP-HA-IκB(S32,36A; *IκB*). The levels of mRNA were determined by real-time PCR as in Fig. 1 and calculated as relative to the average in the absence of IκB. *, $P < 0.05$; **, $P < 0.01$, significantly different from corresponding cells in the absence of IκB (Student's *t* test). *B,* HN13(TVA) keratinocytes were transiently cotransfected with plasmid vectors driving expression of DsRed2 (*control*), Snail1, Snail2, or RelA, with (+) or without (−) IκB, and a NFκB luciferase reporter plasmid. Luciferase activities were measured and reported relative to control without IκB. Data are compiled from two to six independent experiments. ***, $P < 0.001$, significantly different from control without IκB (Student's *t* test). *C,* cells were transfected as in *B*, but the luciferase reporter gene was driven by the human E-cadherin promoter (−400 to +127).

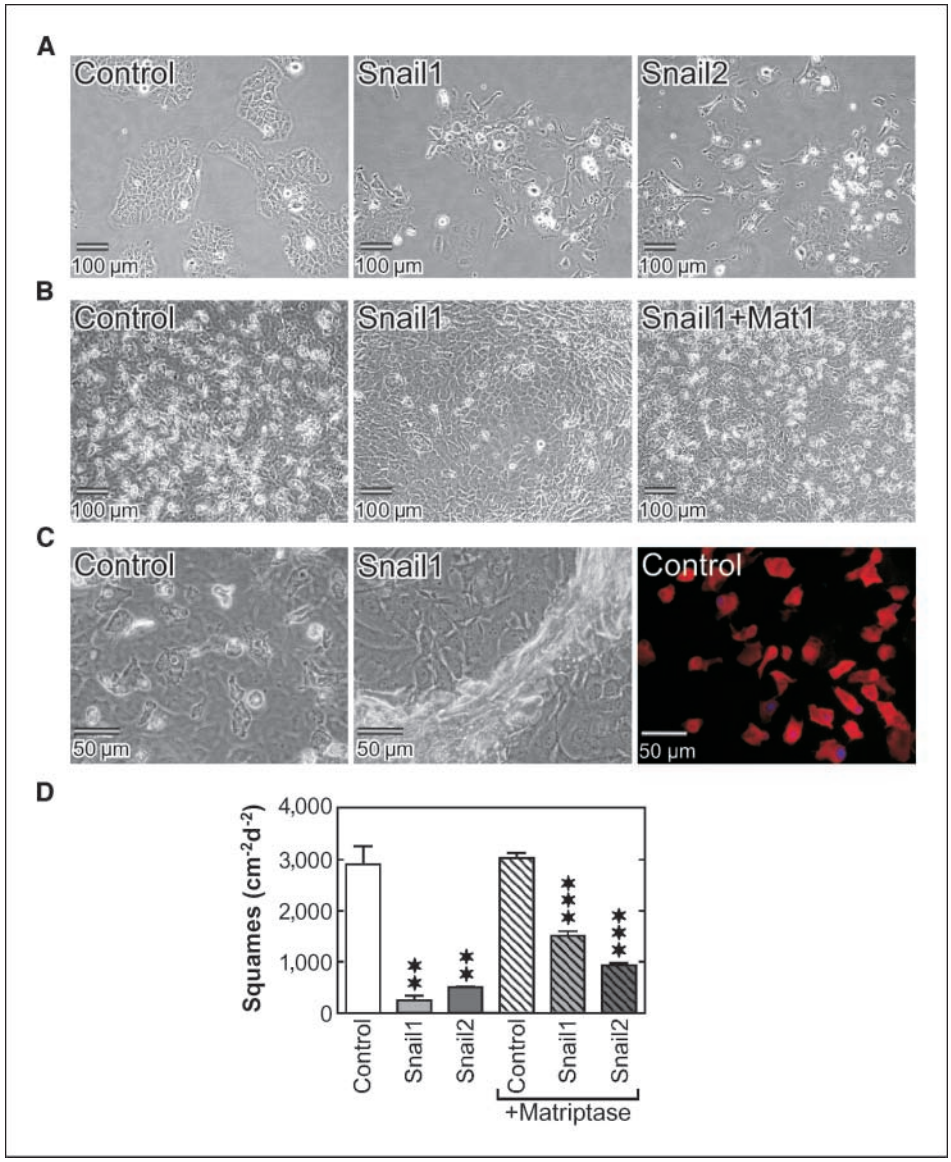

Figure 3. Snails inhibit terminal differentiation of oral keratinocytes. *A,* phase-contrast photomicrographs of control, Snail1-transduced, or Snail2-transduced HN13(TVA) cells, taken after 3 d in culture, showing the scattering effect of Snail expression. *B,* after 14 d, numerous detached squames are evident in phase-contrast micrographs of control cultures (*left*), whereas Snail expression diminishes this effect (*middle*), and matriptase-1 (*Mat1*) expression restores their appearance in Snail-expressing cells (*right*). *C,* higher-magnification images of day 14 cultures showing, in phase contrast, detached squames above the focal plane of a control HN13(TVA) monolayer (*left*) and an upper cell layer in Snail1-expressing cells (*middle*), and a fluorescent image of detached squames from control HN13(TVA) monolayers (days 10–14), lysed in 1% SDS and stained with DAPI (*blue*) to visualize nuclei and with Evans blue (*red*) to visualize SDS-insoluble protein. *D,* quantitation of squames shed from control HN13(TVA) cultures and those expressing Snail1, Snail2, and/or matriptase-1. SDS was added to culture supernatants (days 10–14) to a concentration of 1% (w/v) to lyse nonsquame cells and debris, and then squames were counted in a Beckman Coulter Vicell cell counter. *Columns,* mean from three independent experiments; *bars,* SE. **, $P < 0.01$, significantly lower mean in Snail-expressing cells than in control cells (Student's *t* test). ***, $P < 0.001$, significantly higher mean in cells expressing both matriptase and Snail than in the corresponding cells expressing Snail only (Student's *t* test).

faithfully replicated Snail1 up-regulation, it was tested in the well-characterized MDCK cell line. Dependent on treatment with 4OHT, MDCK cells stably expressing Snail1-ERT2 repressed E-cadherin promoter activity, decreased the amount of endogenous E-cadherin made by those cells (Supplementary Fig. S2), redistributed E-cadherin from the plasma membrane, acquired an epithelial-mesenchymal transition–like morphology, and invaded a collagen matrix from preformed cysts (Supplementary Fig. S3). Thus, the activation of Snail1-ERT2 by 4OHT had the same effects as the ectopic expression of Snail genes in MDCK on E-cadherin, cell morphology, and invasion (3, 10).

Identification of Snail1-regulated genes in oral keratinocytes. To identify Snail1-regulated genes in oral keratinocytes, mRNAs in 4OHT- and solvent-treated HN13/Snail1-ERT2 cells were compared on cDNA microarrays, and genes of interest were validated as Snail1 targets by real-time PCR analysis of cDNAs (Supplementary Table S2). Several genes regulated by Snail1 fell into two groups: epithelial-specific genes that were down-regulated and inflammation-associated genes that were up-regulated. Snail1 down-regulated the epithelial cell-cell adhesion proteins of adherens and tight junctions, E-cadherin and claudin-1, respectively, as has been reported elsewhere (3). Another adherens junction molecule, EpCAM (TACSTD1), was also markedly down-regulated. Matriptase-1 (ST14), a cell-surface serine proteinase required for complete terminal differentiation of keratinocytes (11), was down-regulated, as was ELF3, an epithelial-specific transcription factor required for the expression of genes such as small proline-rich proteins, transglutaminase-3, and profilaggrin during terminal differentiation of keratinocytes (12, 13). Proinflammatory genes whose expression was up-regulated by Snail1 activity included *interleukin-1α* (*IL1a*), *interleukin-1β* (*IL1b*), *interleukin-6* (*IL6*), *interleukin-8* (*IL8*), *CXCL1*, and the prostaglandin synthetic enzyme *cyclooxygenase-2* (*COX2*; *PTGS2*). Control cells expressing EGFP-ERT2 showed that the altered expression of all genes was due to Snail1, and not to 4OHT or the ERT2 moiety.

To ensure that the genes identified by 4OHT induction were genuine targets of Snail1, their regulation was validated

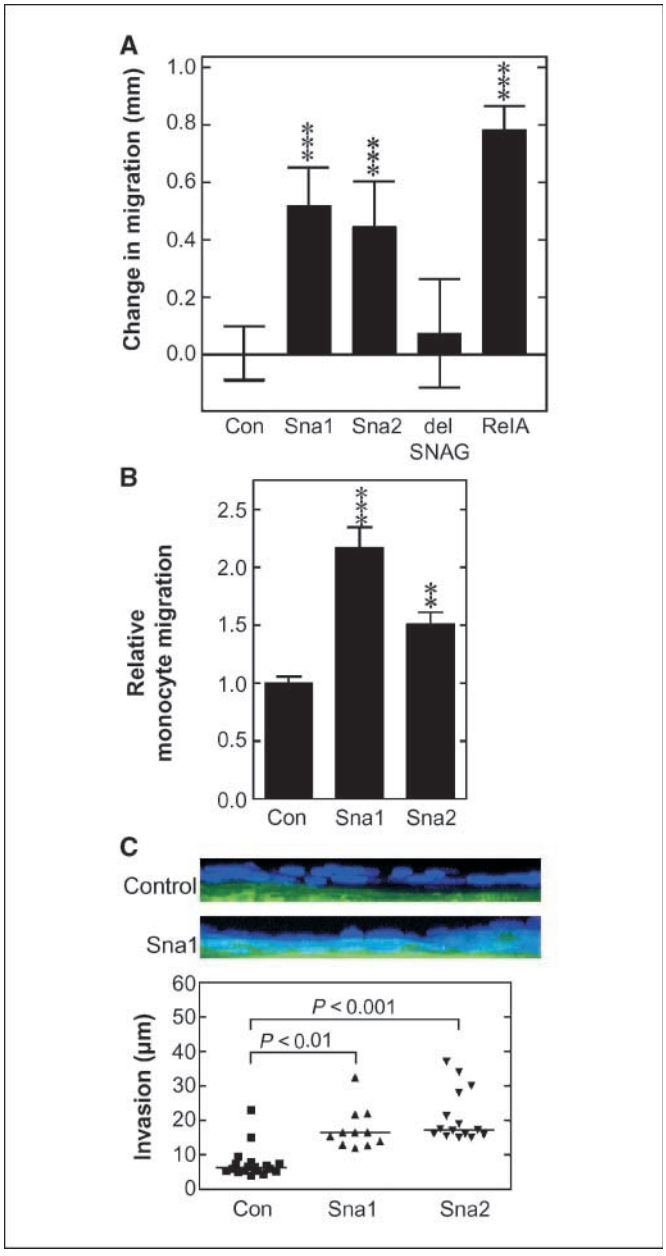

Figure 4. Snails promote migration and invasion of keratinocytes and monocytes. HN13(TVA) keratinocytes were transduced with retroviral vectors expressing Snail1 (*Sna1*), Snail2 (*Sna2*), Snail1-delSNAG (*delSNAG*), or RelA, as described in Fig. 1. *A,* keratinocyte migration assayed by a 16-h scratch type assay. *Columns,* mean from a representative experiment; *bars,* SD. *B,* the migration of THP-1 monocytes across a porous filter barrier toward medium conditioned by control or Snail-transduced HN13(TVA) cells was measured. *Columns,* mean from three independent experiments; *bars,* SE. **, $P < 0.01$; ***, $P < 0.001$, significantly different from the control (Student's *t* test). *C,* invasion of a high-density fluorescent collagen fiber network by control or Snail-expressing HN13(TVA) cells was measured by leading edge analysis of confocal micrographs. Representative fields showing the DAPI-stained nuclei of the cells (*blue*) penetrating the collagen matrix (*green*). The distance invaded by the cells at the invasive front is plotted for control HN13(TVA) cells and those expressing Snail1 or Snail2. **, $P < 0.01$; ***, $P < 0.001$, median significantly different from that of control cells (Mann Whitney *U* test).

independently by transducing HN13(TVA) cells with RCAS retroviral vectors (Supplementary Fig. S4). In each case, Snail1 caused an increase or decrease in expression corresponding to the 4OHT induction results, as determined by real-time PCR (Fig. 1). ELF3, matriptase-1, and EpCAM were strongly repressed in HN13 cells by both Snail1 and Snail2 by a greater extent than E-cadherin and claudin-1 (Fig. 1*A*). This repression was dependent on the presence of the SNAG domain. RelA had no effect on E-cadherin mRNA levels in these cells. Up-regulation of proinflammatory genes was also confirmed using a virally transduced Snail1 gene (Fig. 1*B*), with *COX2, IL6, IL8*, and *CXCL1* being particularly strongly up-regulated (4- to 8-fold). Snail2 induced these same genes. Increases in secreted proinflammatory protein levels were confirmed by immunoassays of the culture supernatants (Supplementary Table S3). Expression of IL6, IL8, and CXCL1 were similarly strongly induced in A431 keratinocytes, derived from an epidermoid SCC, but not in HaCaT non-SCC keratinocytes (Supplementary Table S4).

The role of NFκB activity in Snail induction of proinflammatory genes. The proinflammatory cytokine genes up-regulated by Snails are also induced in keratinocytes by NFκB (ref. 14; Fig. 1; Supplementary Table S4), prompting us to search for a relationship between the two factors. Expression of the NFκB inhibitor IκB inhibited NFκB activity (Fig. 2*B*; Supplementary Fig. S5) and expression of IL6, IL8, and CXCL1, having a greater effect in Snail-induced cells than in control cells (Fig. 2*A*), whereas COX2 was not consistently affected. A reduced expression of Snail genes in IκB-expressing cells may have contributed to this down-regulation of Snail-induced genes (Supplementary Table S5). In cotransfection experiments, expression of an NFκB reporter gene was induced weakly by Snail2 (<2-fold) and not at all by Snail1 under conditions in which RelA caused a strong response (Fig. 2*B*). The basal reporter gene activity was dependent on endogenous NFκB in the cells, as shown by the ability of IκB to inhibit it. In parallel experiments, Snail1 and Snail2 were able to repress transcription from both human (Fig. 2*C*) and mouse (not shown) E-cadherin promoters by 50% to 75%. Overall, the data in Figs. 1 and 2 suggest that NFκB activity might be necessary for strong Snail-induced expression of IL6, IL8, and CXCL1, but that the induction is not mediated by an increase in NFκB activity. NFκB is less important for COX2 induction.

Snails impair terminal differentiation and induce a proinvasive, proinflammatory phenotype. Terminal differentiation is impaired in neoplasms of stratified squamous epithelia, and their progression to malignancy is associated with invasion of the underlying extracellular matrix and an influx of inflammatory cells. Thus, it was of interest to determine the effects of Snail expression on these characteristics. Snail1 and Snail2, but not RelA, produced similar morphologic changes in HN13(TVA) keratinocytes. At low cell density, scattering and elongated projections at the edges of colonies were common (Fig. 3*A*). HN13(TVA) can terminally differentiate and shed squames into the supernatant 10 to 14 days after plating, a process that Snail1 and Snail2 inhibited by >80% (Fig. 3*B–D*), instead causing cells to pile up (Fig. 3*C*). The squames shed by control HN13(TVA) maintained their structure in 1% SDS (Fig. 3*C*), indicating a mature, highly cross-linked cytoskeletal structure. Both nucleated and nonnucleated squames were represented, in a ratio of ~2:1, although many nuclei stained weakly with DAPI, suggesting that their DNA was undergoing degradation. On dissolution of both control and Snail-transduced cultures with 1% SDS, squames were released, identifying the final process of keratinocyte terminal differentiation, desquamation, as being inhibited by Snails. Interestingly, reexpression of matriptase-1 (2- to 3-fold above control levels, as measured by reverse

transcription/real-time PCR), disruption of whose gene inhibits desquamation in humans and mice (15), partially restored desquamation in Snail-expressing keratinocytes (Fig. 3*D*).

Expression of Snail1, Snail2, or RelA increased the migration of HN13(TVA) cells (Fig. 4*A*). Additionally, THP-1 monocytes showed a 50% to 120% higher migration toward medium conditioned by Snail-expressing keratinocytes than that of control keratinocytes (Fig. 4*B*). Blocking IL1, IL6, and IL8/CXCL1 activity with inhibitory concentrations of IL1 receptor antagonist, antibodies to IL6, and CXCR2 inhibitors (SB225002 or antibodies), respectively, did not inhibit the Snail-induced THP-1 migration, indicating that another molecule(s) is responsible. Moreover, Snail expression doubled the rate of invasion by keratinocytes into a high-density fibrillar interstitial collagen matrix (Fig. 4*C*).

Discussion

We have shown that the expression of Snail transcriptional repressors in keratinocytes impairs their differentiation into shed squames, an effect that can be rescued by forced reexpression of the Snail down-regulated gene matriptase-1. Gene targeting of claudin-1 (16) and matriptase-1 (11) has shown them to be required for complete terminal differentiation of keratinocytes, with null mice dying from impaired epidermal barrier formation. Recently, a human genetic disease characterized by impaired desquamation was shown to be caused by a mutation in matriptase-1 (15). Another Snail target, ELF3, drives the expression of terminal differentiation proteins (12, 13). Thus, down-regulation of these genes is consistent with the failure to shed squames in Snail-expressing keratinocytes. It has previously been shown that expression of Snail2 in keratinocytes of the upper airway tract increases with malignancy and local recurrence following treatment (4). Hyperplasia of stratified squamous epithelia is accompanied by impaired terminal differentiation, increasing the accumulation of viable keratinocytes in the tissue, and thus it is likely that Snail2 contributes to this process during HNSCC formation.

Inflammation is commonly associated with cancer, and the up-regulation of IL1a, IL6, and IL8 (2) has been observed in HNSCCs, with enhanced NFκB activity being implicated as mediating this effect (14). We have shown here that Snails can also up-regulate these proinflammatory cytokines in oral keratinocytes. Snails are known to be repressors and have not been reported to act as transcriptional activators. There are no consensus Snail-binding E-boxes within 300 bp upstream of the transcription start site of the *COX2, IL6, IL8*, or *CXCL1* genes, whereas the *ELF3, EpCAM*, and *matriptase-1* genes, like the *E-cadherin* gene, have at least two sites. Deletion of the SNAG domain reduced the up-regulation of the proinflammatory genes, suggesting that recruitment of corepressors was important for the effect. Thus, the up-regulation of the proinflammatory genes is likely to be an indirect effect (e.g., through down-regulation of a repressor of these genes or competition for corepressors). The poor induction of NFκB reporter gene activity by Snail1 (no induction) and Snail2 (1.7-fold) suggests that the up-regulation of the strongly induced genes is not mediated by increasing NFκB activity, although NFκB might be necessary for cytokine induction by Snails. Thus, two pathways may have evolved to up-regulate these molecules in keratinocytes, presumably to respond to different stimuli. In contrast to the cytokines, COX2 expression was weakly stimulated by RelA, whereas it was strongly stimulated by Snails. The mitogen-activated protein kinase pathways seem to be the dominant routes of up-regulating COX2 expression in human keratinocytes in response to cytokines, with NFκB playing a minor role (17), unlike mouse keratinocytes and other human cell types. COX2, like Snail2, predicts poor outcomes for HNSCC patients (18).

Recently, microarray analysis of gross epidermis was used to identify Snail2 targets in normal adult mouse epidermal cells (19). Of the Snail targets mentioned here, only EpCAM was modified in mouse epidermis by disruption of both Snail2 loci (1.5-fold higher in Snail2-null mice). The levels of Snails in normal keratinocytes might be too low to substantially up-regulate or down-regulate the target genes identified in this study. However, in keratinocytes that have undergone malignant transformation or wounding (4, 20), Snail expression is raised and the regulation of targets identified in this study would take place, thereby facilitating migration, invasion, and the recruitment of leukocytes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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