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The transcription factor Snail enhanced the degradation of E-cadherin and desmoglein 2 in oral squamous cell carcinoma cells

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ABSTRACT

Epithelial–mesenchymal transition (EMT), a key process in the tumor metastatic cascade, is characterized by the loss of cell–cell junctions and cell polarity as well as the acquisition of migratory and invasive properties. However, the precise molecular events that initiate this complex EMT process are poorly understood. Snail is a regulator of EMT that represses E-cadherin transcription through its interaction with proximal E-boxes in the promoter region of target genes. To investigate the role of Snail in EMT, we generated stable Snail transfectants using the oral squamous cell carcinoma cell line HSC-4 (Snail/HSC-4). Snail/HSC-4 cells had a spindle-shaped mesenchymal morphology, and enhanced migration and invasiveness relative to control cells. Consistent with these EMT changes, the downregulation of epithelial marker proteins, E-cadherin and desmoglein 2, and the upregulation of mesenchymal marker proteins, vimentin and N-cadherin were detected. Despite these observations, the mRNA levels of E-cadherin and desmoglein 2 did not decrease significantly. Although E-cadherin and desmoglein 2 proteins were stable in parental HSC-4 cells, these proteins were rapidly degraded in Snail/HSC-4 cells. The degradation of E-cadherin, but not desmoglein 2, was inhibited by dynasore, an inhibitor of dynamin-dependent endocytosis. Therefore, in HSC-4 cells Snail regulates levels of these proteins both transcriptionally and post-translationally.

1. Introduction

Among oral malignancies, oral squamous cell carcinoma (OSCC) is the most common cause of deaths worldwide, with an estimated 300,000 new cases each year [1]. Despite advances in the diagnosis and treatment of OSCC the prognosis remains poor, mainly because of its high recurrence rate and tendency to metastasize. Therefore, further understanding of the development and progression of OSCC as well as the discovery of new molecular targets are of great importance.

Epithelial–mesenchymal transition (EMT) is a complex process by which epithelial cells lose their polarity and reorganize their cytoskeleton, while also acquiring a mesenchymal phenotype and increased motility [2,3]. In addition to tissue remodeling, organ development, and wound healing, EMT plays a critical role in cancer progression [4–7]. Loss of a polarized epithelial phenotype and acquisition of mesenchymal characteristics endow tumor cells with the potential to invade and metastasize.

Epithelial cells are connected together by the epithelial junctional complex, which consists of tight junctions, adherens junctions, and desmosomes. E-cadherin is a component of the adherens junction and is involved in formation and maintenance of the epithelial structure [8]. Desmoglein 2 is a component of the desmosomes and is expressed ubiquitously in epithelial and nonepithelial cells [9]. E-cadherin and desmoglein 2 are members of the cadherin family of the cell–cell adhesion molecules.

Snail belongs to the Snail superfamily of zinc finger transcription factors [10]. Snail and Slug, a related superfamily member, are expressed in the early mesoderm and neural crest during development [11–13]. These two zinc finger transcription factors repress E-cadherin transcription through an interaction of their C-terminal region with a 5′-CACCTG-3′ sequence (referred as E-box) in the cadherin promoter [14,15]. Correlative studies have shown that there is an inverse relationship between E-cadherin expression and Snail expression in human samples [16].

Although possible involvement of Snail or Slug in transforming growth factor β1-mediated EMT of OSCC has been reported

Abbreviations: EMT, epithelial–mesenchymal transition; OSCC, oral squamous cell carcinoma.

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recently [17,18], the role of Snail in these processes are still unclear. Therefore, we ectopically expressed Snail in HSC-4 cells, an OSCC cell line, and then analyzed the consequences of Snail expression on the cells.

2. Materials and methods

2.1. Cell lines and transfection

HSC-4 cells, a squamous cell carcinoma line that originated from human tongue cancer, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. HSC-4 cells were transfected with 10 μg of HA-tagged human Snail plasmid (pC-SnailHA) or control plasmid encoding HA-tagged GFP (pC-GFPHA) using the calcium phosphate method, as described previously [19,20].

2.2. Antibodies

Mouse monoclonal antibodies against E-cadherin, vimentin, and Snail were purchased from BD Biosciences (Lexington, KY), Zymed Laboratories (South San Francisco, CA), and Cell Signaling Technology (Danvers, MA), respectively. DECMA-1, a monoclonal antibody to extracellular domain of E-cadherin was described previously [21]. Mouse monoclonal antibody against desmoglein 2 was purchased from Progen Biotechnik GmbH (Heidelberg, Germany). Mouse monoclonal antibody against vinculin was purchased from Sigma (St. Louis, USA). Rat monoclonal antibody against HA was purchased from Roche Applied Science (Mannheim, Germany). All secondary antibodies were obtained from Jackson Immuno Research Laboratories (West Grove, PA).

2.3. RT-PCR analysis

Total RNA was extracted from the cells using the Isogen kit (Wako, Osaka Japan) and then reverse transcribed using Rever Tra Ace (Toyobo, Osaka, Japan). The resulting cDNAs were used as templates for specific PCR reactions using GoTaq DNA polymerase (Promega, Madison, WI). The PCR conditions were optimized for each primer pair as described previously [22]. The following primer combinations were used: E-cadherin, sense (GACACCCGATTCAAAGTGGG) and antisense (GTCTCTCTTCTGTCTTCTGAG); Snail, sense (ACTACAGCGAGCTGCAGG) and antisense (GTGTGGCTTCGGATGTGC); desmoglein 2, sense (GAAGCAAGAGATGGCAATGG) and antisense (GCCAGCCAATTATCAGAACC); vimentin, sense (AATGGCTCGTCACCTTCGTGAAT) and antisense (CAGATTAGTTTCCCTCAGGTTCAG); β-actin, sense (CAAAGACCTG TACGCCAACAC) and antisense (CATACTCCTGCTTGCTGATCC).

2.4. Invasion assay

The invasive activity of the cells was measured using BioCoat MatriGel Invasion Chambers (BD Biosciences) according to the manufacturer's protocol. The lower compartment was filled with DMEM medium containing 10% FBS as a chemoattractant. The upper compartments were seeded 5×10^5 cells in serum-free DMEM medium. After 22 h of incubation at 37 °C, the migrated cells were collected and counted.

2.5. Wound healing assay

The HSC-4 and Snail/HSC-4 cells were plated on Falcon 3001 dishes containing 10% FBS in DMEM and allowed to grow to confluency. The cell monolayer was wounded by manual scratching with a pipette tip, washed with PBS, then incubated for 12 h. A phase contrast microscope was used to photographed the cells at 0 and 12 h after making the wound scratch.

2.6. Immunoblotting

For immunoblot analysis, cells were boiled for 5 min in SDS gel sample buffer. Proteins were separated by either 5% or 8% polyacrylamide gel electrophoresis, and were transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in PBS, and were incubated with specific primary antibodies followed by treatment with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). After washing with PBS containing 0.1% Tween-20, protein bands were visualized by enhanced chemiluminescence (ECL) (Amersham International, Little Chalfont, UK) as described previously [20].

2.7. Immunofluorescence staining

For immunofluorescence, cells were grown on coverslips, fixed with 3% paraformaldehyde in PBS (for 20 min at room temperature), and permeabilized with 0.1% Triton X-100. The coverslips were immunostained with primary and secondary antibodies as described previously [20]. Cells were analyzed using a conventional Olympus fluorescence microscope (Tokyo, Japan). For the detection of cell surface E-cadherin and desmoglein 2 by immunofluorescence staining, living cells were incubated with primary antibodies raised against the extracellular portions of E-cadherin or desmoglein 2 (DECMA-1 or anti-desmoglein 2) for 30 min at 4 °C. After washing, the cells were incubated for 0 or 2 h at 37 °C to allow internalization of these proteins. The cells were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature and then incubated with secondary antibodies. The cells were not permeabilized by Triton X-100 to allow the detection of these proteins on the cell surface.

2.8. Cycloheximide-chase experiments

Cells that were grown for 24 h in DMEM medium containing 10% FBS were cultured for 0, 6, 12, 18, and 24 h in the presence of cycloheximide (10 μM) to prevent protein synthesis. Whole cell lysates were resolved using SDS–PAGE and subjected to immunoblot analysis.

3. Results

3.1. Snail induces morphological changes and promotes motility and invasiveness of HSC-4 cells

HSC-4 cells, a cell line derived from human OSCC, have epithelial properties including brick stone morphology. We introduced an expression vector encoding Snail (Snail/HSC-4), or GFP as a control (GFP/HSC-4), into HSC-4 cells to generate stable transfectants after selection in G418. GFP/HSC-4 cells retained the same epithelial characteristics as parental HSC-4 cells, while Snail/HSC-4 cells exhibited a spindle shape (Fig. 1A). Ectopic expression of Snail induced morphological changes that were characteristic of EMT.

Cells undergoing EMT lose cell–cell adhesion and increase their motility. Wound-healing assays demonstrated significant differences in directional cell migration and motility between parental HSC-4 cells and Snail/HSC-4 cells (Fig. 1B). Snail/HSC-4 cells closed the wound area faster than HSC-4 cells (Fig. 1B). In invasion assays, Snail/HSC-4 cells invaded about 7.6 times more than HSC-4 cells (Fig. 1C). These results paralleled the results of the wound-healing assay, further supporting the hypothesis that Snail drives EMT in OSCC.

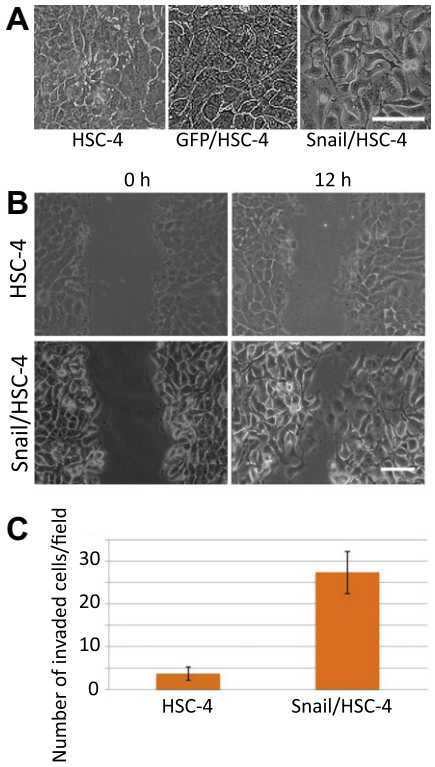

Fig. 1. Stable Snail transfectants of HSC-4 cells display mesenchymal characteristics. (A) Parental HSC-4 cells and HSC-4 cells transfected with an expression vector encoding for GFP (GFP/HSC-4 cells) show a typical epithelial cell morphology, while HSC-4 cells transfected with an expression vector encoding Snail (Snail/HSC-4 cells) show a fibroblastic morphology. Bar, 50 μm. (B) Wound healing assays showed that Snail/HSC-4 cells had a significantly greater migration activity than parental HSC-4 cells. Bar, 50 μm. (C) Snail/HSC-4 cells showed significantly increased invasion than parental HSC-4 cells.

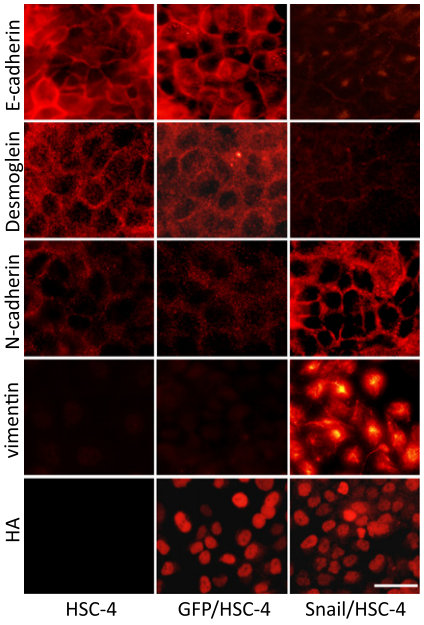

Fig. 2. Immunostaining revealed that Snail expression downregulates E-cadherin and desmoglein 2 expression, while up-regulating N-cadherin and vimentin expression. Bar, 50 μm.

3.2. Snail expression decreased the expression of epithelial markers and increased the expression of mesenchymal markers

Next, we determined expression and localization of epithelial markers such as E-cadherin and desmoglein 2 using immunofluorescence staining. In HSC-4 cells and GFP/HSC-4 cells, E-cadherin and desmoglein 2 were strongly stained and clearly localized to the cell membrane (Fig. 2). On the other hand, mesenchymal markers such as N-cadherin and vimentin were only weakly expressed in HSC-4 cells and GFP/HSC-4 cells. In Snail/HSC-4 cells, the expression of E-cadherin and desmoglein 2 were reduced, while the expression of N-cadherin and vimentin were increased (Fig. 2).

Immunoblot analysis supports the above observation that HSC-4 cells express epithelial characteristics, namely high expression of E-cadherin and desmoglein 2 and low expression of N-cadherin and vimentin (Fig. 3A). Although GFP/HSC-4 cells showed essentially the same characteristics as parental HSC-4 cells, Snail/HSC-4 cells exhibited quite different characteristics (Fig. 3A). In Snail/HSC-4 cells, the expression of E-cadherin and desmoglein 2 were downregulated, while N-cadherin and vimentin expression were upregulated (Fig. 3A).

Comparison of the gene expressions of Snail/HSC-4 cells and parental HSC-4 cells using Agilent Whole Human Genome microarrays revealed moderately decreased expression of E-cadherin and desmoglein 2 and significantly increased expression of N-cadherin and vimentin (Table 1). Increased expression of N-cadherin and vimentin was consistent with the immunofluorescence and immunoblotting data. RT-PCR was used to confirm the

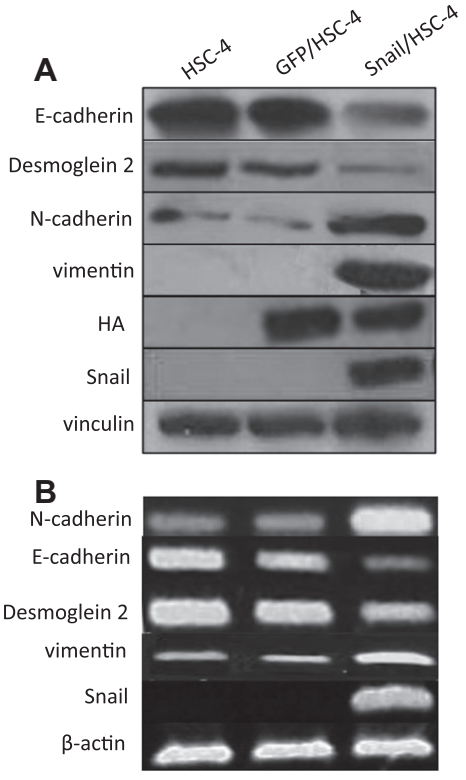

Fig. 3. EMT is induced in Snail/HSC-4 cells. (A) Immunoblot analysis revealed that Snail expression in HSC-4 cells decreased the expression of epithelial markers (E-cadherin and desmoglein 2) and increased the expression of mesenchymal markers (N-cadherin and vimentin). Vinculin was used as a loading control. (B) RT-PCR analysis of E-cadherin, desmoglein 2, and vimentin mRNA in HSC-4, GFP/HSC-4, and Snail/HSC-4 cells. β-actin was used as an internal control.

microarray data. Although the expression of mRNAs for E-cadherin and desmoglein 2 were slightly decreased in Snail/HSC-4 cells, the

Table 1
Relative expression levels of epithelial and mesenchymal markers in Snail/HSC-4 cells.

	Immunoblot	Microarray	RT-PCR*
E-cadherin	0.20	0.46	0.37 ± 0.22
Desmoglein 2	0.34	0.85	0.56 ± 0.21
N-cadherin	5.75	5.47	1.92 ± 0.41
Vimentin	29.0	6.91	1.60 ± 0.28

The expression levels were determined using ImageJ (National Institutes of Health). The data are presented as the relative intensity of the bands from Snail/HSC-4 cells to that of parental HSC-4 cells. *Values are the mean ± the S.E. for triplicate determinations.

mRNA expression for vimentin was increased (Fig. 3B). Thus, there was a discrepancy between E-cadherin and desmoglein 2 mRNA expression levels and their protein levels. N-cadherin and vimentin mRNA and protein levels were correlated.

3.3. E-cadherin and desmoglein 2 proteins were rapidly degraded in Snail/HSC-4 cells

We next examined the rate of protein turnover in Snail/HSC-4 cells to determine the cause of the significantly reduced protein levels of E-cadherin and desmoglein 2 despite only a slight reduction of their mRNA levels. HSC-4 cells and Snail/HSC-4 cells were treated with cycloheximide (20 μM) to prevent protein synthesis and then subjected to immunoblotting to determine the expression of E-cadherin or desmoglein 2 (Fig. 4A). Quantification of the signals showed that E-cadherin and desmoglein 2 proteins rapidly decreased in Snail/HSC-4 cells compared to HSC-4 cells (Fig. 4B). The ectopic expression of Snail in HSC-4 cells increased the instability of E-cadherin and desmoglein 2, which was due to the rapid degradation of these proteins in Snail/HSC-4 cells.

To determine the kinetics of internalization, E-cadherin and desmoglein 2 proteins on the cell surface were labeled with antibodies that were raised against the extracellular part of these proteins. The labeled cells were then incubated for 2 h at 37 °C to allow the proteins to be internalized. As shown in Fig. 4C, a significant amount of E-cadherin and desmoglein 2 remained on the cell surface of HSC-4 cells. In contrast, almost all of E-cadherin and desmoglein 2 on Snail/HSC-4 cells was internalized. These findings were consistent with the rapid degradation of E-cadherin and desmoglein 2 following ectopic expression of Snail.

Internalization of E-cadherin can occur through dynamin-dependent and dynamin-independent pathways [23]. Although the mechanism of desmoglein 2 internalization is currently unknown, antibody-induced endocytosis of desmoglein 3 has been shown to be mediated by a dynamin-independent mechanism [24]. To examine the requirement of endocytosis for protein degradation, we treated the cells with dynasore, which is a potent inhibitor of dynamin-dependent endocytosis [25]. As shown in Fig. 4B, accelerated degradation of E-cadherin, but not that of desmoglein 2, in Snail/HSC-4 cells was suppressed with dynasore treatment. The decreased E-cadherin expression in Snail/HSC-4 cells was likely due to the increased protein degradation through the dynamin-mediated internalization.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates a broad range of cellular responses [26]. TGF-β is the major mediator of EMT and induces expression of Snail [27] and Slug [28]. To examine the above observations are applicable to TGF-β-induced EMT, HSC-4 cells were incubated with TGF-β and expression of Snail and E-cadherin was determined. RT-PCR analysis revealed 2.3-fold increase of Snail mRNA and slight decrease (0.7-fold increase) of E-cadherin mRNA under the conditions used (data not shown). Importantly, the amounts of the cell surface E-cadherin decreased upon TGF-β incubation and the TGF-β-induced decrease of the cell surface E-cadherin was inhibited by pre-incubation of the cells with dynasore (Fig. 4D).

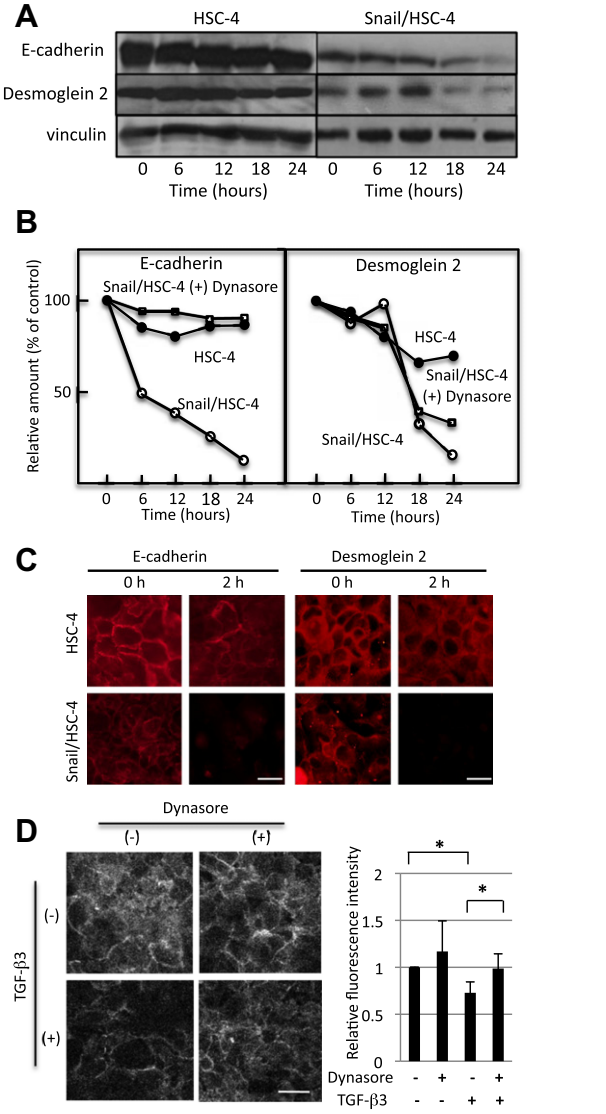

Fig. 4. Rapid degradation of E-cadherin and desmoglein 2 in Snail/HSC-4 cells. (A) HSC-4 cells and Snail/HSC-4 cells were treated with cycloheximide (20 μM) for the indicated time periods. Whole cell lysates were subjected to immunoblot analysis with E-cadherin and desmoglein 2 antibodies. (B) Quantification of the signals showed that E-cadherin and desmoglein 2 in Snail/HSC-4 cells decreased rapidly compared to HSC-4 cells. Dynasore (50 μM) significantly depressed the degradation of E-cadherin but not desmoglein 2. ImageJ (National Institutes of Health) was used to quantify the protein levels. Data are normalized to the protein levels at time 0. (C) Cells were incubated with antibodies raised against the extracellular portion of E-cadherin or desmoglein 2 for 30 min at 4 °C. After washing to remove unbound antibodies, cells were incubated for an additional 2 h at 37 °C, fixed with 3% paraformaldehyde, and then E-cadherin or desmoglein 2 on the cell surface was detected. (D) TGF-β reduces cell surface E-cadherin whereas dynasore stabilizes cell surface E-cadherin. Cells were incubated with dynasore (50 μM) for 30 min and then TGF-β3 (100 pM) for 16 h in the presence of dynasore. (Left) E-cadherin on the cell surface was detected as above using a Zeiss LSM 700 laser-scanning confocal microscope. Bar, 50 μm. (Right) The fluorescence intensity of E-cadherin at the cell–cell junctions was recorded from 5 cells in independent 3 visual fields and quantified using ZEN lite 2011 software. Data are normalized to the intensity of the cells without TGF-β and dynasore treatment. Values are the mean ± the S.E. for determinations from three independent experiments. Asterisks indicate a statistical difference from the corresponding cells ($P < 0.05$) (Student's t-test).

4. Discussion

In the present study, we showed that ectopic expression of Snail in HSC-4, a human OSCC cell line, induced changes that were characteristics of EMT. The observed changes included the acquisition of fibroblast-like morphology and invasive phenotypes. In addition, these cells had decreased expression of E-cadherin and desmoglein 2 (desmosomal cadherins), and the increased expression of N-cadherin and vimentin (mesenchymal markers). Immunofluorescence staining and immunoblot analysis showed significantly reduced expression of E-cadherin and desmoglein 2 proteins. RT-PCR analysis and DNA microarray analysis, however, revealed only a slight decrease in E-cadherin and vimentin mRNAs. Although previous microarray analysis in Madin–Darby canine kidney (MDCK) epithelial cells showed that Snail downregulated the transcription of epithelial markers, including E-cadherin and desmoglein 2 [29], in this current study Snail did not significantly affect the transcription of E-cadherin and desmoglein 2 in HSC-4 cells. Our data showed that the degradation of E-cadherin and desmoglein 2 was dramatically enhanced by the expression of Snail. Similarly, the internalization of these proteins was also enhanced by Snail expression. Thus, the expression of Snail destabilizes E-cadherin and desmoglein 2, possibly by enhancing the internalization of these proteins.

The degradation of E-cadherin, but not desmoglein 2, in the cells that expressed Snail was suppressed by the inhibition of dynamin-dependent endocytosis. This finding suggested that Snail accelerated the clathrin-mediated, dynamin-dependent endocytosis of E-cadherin, and it is not the same mechanism responsible for the degradation of desmoglein 2. The results for desmoglein 2 largely recapitulate a previous observation of antibody-induced internalization of desmoglein 3 via a dynamin-independent mechanism [24]. Therefore, Snail appears to accelerate both dynamin-dependent and dynamin-independent pathways.

Previous knockdown studies of sea urchin embryos demonstrated that Snail not only repressed the transcription of cadherin, but was also required for endocytosis of cadherin [30]. Previous reports have shown that epithelial cells at the wound margins of the corneal epithelium express Slug, and have a reduced expression and impaired trafficking of E-cadherin from the cell membrane to the cytosol, which were linked to the development of a migratory phenotype [31]. Intracellular redistribution of desmoglein in stable Slug transfectants of the rat NBT-II bladder carcinoma cell line has also been reported [32]. Thus our observation that Snail expression enhances internalization of E-cadherin and desmoglein 2 is not specific for HSC-4 cells. Although these papers analyzed the distribution of E-cadherin and desmoglein in these cells, to the best of our knowledge our study is the first report to show that Snail decreases the stability of these proteins by facilitating their internalization. Currently, we do not know the mechanism by which Snail enhances the internalization and degradation of E-cadherin and desmoglein 2 in HSC-4 cells. It has been shown that loss or reduced expression of protein phosphatase 2A in human breast carcinomas is correlated to the diminished function of E-cadherin via endocytosis [33]. Further studies are required to determine whether protein phosphatase 2A is also a target of Snail. In our previous study we showed that 30–40% of the ectopically expressed E-cadherin in Snail-expressing MDCK cells was localized to the intracellular compartments and concentrated in the perinuclear region [19], suggesting that the transport of E-cadherin to the cell surface was partially inhibited by Snail expression. The mechanism underlying responsible for the blocked transport of E-cadherin is undefined.

In conclusion, we showed that E-cadherin and desmoglein 2 were significantly decreased in human OSCC cells that expressed Snail. The decreased expression of E-cadherin and desmoglein 2 in these cells was due to protein degradation via dynamin-dependent and dynamin-independent internalization of E-cadherin and desmoglein 2, respectively.

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