Snail Induction of Epithelial to Mesenchymal Transition in Tumor Cells Is Accompanied by MUC1 Repression and ZEB1 Expression*

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E-cadherin protein plays a key role in the establishment and maintenance of adherent junctions. Recent evidence implicates the transcription factor Snail in the blockage of E-cadherin expression in fibroblasts and some epithelial tumor cells through direct binding to three E-boxes in the E-cadherin promoter. Transfection of Snail into epithelial cells leads to a more fibroblastic phenotype. Cells expressing Snail presented a scattered flattened phenotype with low intercellular contacts. Other epithelial markers like Cytokeratin 18 or MUC1 were also repressed. The effects of Snail on MUC1 transcription were mediated by two E-boxes present in the proximal promoter. Snail also induced expression of the mesenchymal markers fibronectin and LEF1 and the transcriptional repressor ZEB1. ZEB1 and Snail had a similar pattern of expression in epithelial cell lines, and both were induced by overexpression of ILK1, a kinase that causes the loss of E-cadherin and the acquisition of a fibroblastic phenotype. Snail overexpression in several cell lines raised ZEB1 RNA levels and increased the activity of ZEB1 promoter. ZEB1 could also repress E-cadherin and MUC1 promoters but less strongly than Snail. However, since ZEB1 expression persisted after Snail was down-regulated, ZEB1 may regulate epithelial genes in several tumor cell lines.

The poor prognosis in epithelial neoplasia is associated with the acquisition of motile or invasive properties by the cancerous cells. This morphological transformation is often referred to as epithelial mesenchymal transition (EMT). 1 EMT was first described in development when it is closely regulated and associated with processes like gastrulation or neuroepithelium formation. Molecular events during EMT include alterations in cell-cell adhesion, cell-substrate interaction, extracellular matrix degradation, and cytoskeleton organization. During EMT, epithelial markers are down-regulated, among them E-cadherin, a protein essential for the establishment of cell-cell adhesion (for review, see Refs. 1–3).

In cancerous cells there is a high correlation between invasion and metastasis and the loss of E-cadherin (4, 5), whereas during gastrulation E-cadherin is down-regulated in progenitor cells in the primitive streak (6). Control of E-cadherin transcription is the main mechanism responsible for the down-regulation of this protein (7, 8). A transcriptional factor called Snail (SNA in humans and Sna in mice) represses E-cadherin transcription in vitro and in vivo by binding to a 5’-CACCTG-3’ sequence of the E-cadherin promoter (9). Transfection of Snail in epithelial cells decreases E-cadherin levels and induces changes resembling EMT (9, 10). In addition, Snail is believed to contribute to the EMT in several experimental models. For instance, during Drosophila gastrulation, Snail function is required for the repression in the mesoderm of genes that are otherwise expressed in the adjacent neuroectodermal regions of the blastoderm (11). Further evidence was obtained from Sna−/− mouse embryos, which show an incomplete EMT: a new mesoderm is formed, but the resulting cells maintain epithelial markers like E-cadherin (12). Other mutants that show defects in EMT and cell migration during gastrulation are fibroblast growth factor receptor 1 (−/−) animals; these defects have been attributed to a severe reduction of Snail expression in the primitive streak accompanied by ectopic expression of E-cadherin (13). Several conditions also induce EMT in epithelial cell lines (3). Snail is involved in a number of processes: for instance, activation of integrin-linked kinase (ILK), which mediates extracellular matrix signals (14, 15), increases Snail promoter activity and down-regulates E-cadherin (16). Finally, increased Snail expression has been described in some carcinogenic cell lines with invasive capacity (10, 17–19).

In this study we have examined with greater detail the changes induced by the ectopic expression of Sna in epithelial cells. In addition to showing the ultrastructural modifications...
observed in cells expressing Snai, we have also characterized several genes that show an altered expression. One of these genes is ZEB1, a transcriptional repressor also capable of blocking the expression of E-cadherin. The data obtained together with data previously reported clearly show that Snai induces a complete EMT.

**MATERIALS AND METHODS**

**Cell Culture**—The generation of Madin-Darby canine kidney (MDCK) cells and HT-29 M6 cells transfected with Sna-HA has been described (9). HT-29 M6 SNA1 and SNA2 correspond to two clones obtained by transfection with a tet-regulated expression vector (tet-off) containing hemagglutinin-tagged mouse Snai cDNA (Snai). MDCK SNA1 and SNA3 are two MDCK Sna-expression clones obtained by transfection of MDCK cells with pRES-neo Sna-HA (9). IEC-18 rat cells transfected with wild-type or negative forms of ILK or with an antisense construct of this kinase were obtained as described previously (14). Other human (HT-29, SW-480, SW-620, ZR-75, T47D, MCF-7, MiaPaca, and RWP-1) or mouse (EpiH and NIH-3T3) cell lines were obtained from our institute Cell Bank. All cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum (Biological Industries) and the standard supplements.

**DNA Constructions and Other Reagents**—Murine ZEB1 cDNA was provided by T. Genetta (Children’s Hospital of Philadelphia, Philadelphia, PA) and cloned into pCDNA3HisC expression vector (Promega) at the XhoI site. ZEB2-C52T expression vector was kindly provided by A. Postigo (Washington University, St. Louis, MO). Sna-HA was cloned into RSV 5 expression vector inserting the 0.9-kb HindIII-NovI fragment from pcDNA3 Sna-HA at the XhoI site of RSV 5. Blunt ends were generated using the Klenow fragment from DNA polymerase (New England Biolabs). Commercial anti-ZEB1 was obtained from Santa Cruz Biotechnology (goat anti-ZEB1 C-20, sc-10570) and monoclonal anti-E-cadherin from Transduction Laboratories (c20820). Antiserum against recombinant mouse Snai was raised in New Zealand rabbits by injecting recombinant mouse Snai and purified by affinity chromatography using recombinant murine Snai.

**Sequencing Techniques and Electron Microscopy**—Postconfluent cells were fixed in 2% glutaraldehyde for 30 min and embedded in EPON (Tousimis Research Corp., Rockville, MD). Semithin and ultrathin sections were obtained and stained using standard procedures. For electron microscopy, ultrathin sections were obtained. For western blot, Northern blots were performed following a standard protocol. Briefly, 12.5 μg of total RNA isolated by guanidine isothiocyanate extraction were separated in a denaturing agarose/formaldehyde gel, visualized with ethidium bromide staining, photographed, and transferred to a Zeta-probe (Bio-Rad) membrane overnight by capillary action. The membranes were washed three times in 50 mM Na2HPO4, 150 mM NaCl, 0.1% SDS, 0.1% Tween 20, dried, and exposed to Hyperfilm MP (Amersham Biosciences). RPS9 and RPS26 served as internal controls.

**Analysis of Transcripts**—Northern blots were performed following a standard protocol. Briefly, 12.5 μg of total RNA isolated by guanidine isothiocyanate extraction were separated in a denaturing agarose/formaldehyde gel, visualized with ethidium bromide staining, photographed, and transferred to a Zeta-probe (Bio-Rad) membrane overnight by capillary action. The next day RNA was RNA was hybridized to a radiolabeled probe using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sense oligonucleotide sequence was 5′-GAGGGGGCGGGTTTTTTGAAACCATATAACCTCCTGCTGCTGGTTCAGGGCCG-3′, where mutared nucleotides are indicated in bold.

**Analysis of Promoter Activity**—Repression of E-cadherin or MUC1 promoter activity was measured by cotransfection with ZEB or Sna constructs in pcDNA3 plasmid (Invitrogen) with pGL3-E-cadherin or -MUC1 promoters in the indicated cell lines. Cotransfections included a Renilla reniformis luciferase plasmid (pRTK-Luc or pRSV-Luc from Promega) to normalize transfection efficiency. Firefly luciferase (Luc) and Renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) 48 h after transfection according to the manufacturer’s instructions. Luciferase activity was normalized by Renilla luciferase activity. The level of luciferase activity was normalized by a luciferase activity standardization with empty vector. Duplicates or triplicates were systematically included, and experiments were repeated at least three times. ZEB1 or MUC1 promoter activity in Sna clones was assayed by cotransfecting varying amounts of pGL3-ZEB1 or -MUC1 promoter reporter and pRTK-Luc and measuring luciferase activity as mentioned above.

**Analysis of Protein Expression**—Cells were seeded on 1-cm diameter cover glasses, left to grow 40–70% confluent, fixed with 4% paraformaldehyde, blocked with 5% milk in phosphate-buffered saline, and probed with different antibodies. Fluorescein isothiocyanate–conjugated anti-goat or anti-rabbit (DAKO) was used as second antibody. Immunofluorescent labeled cells were observed in a fluorescent microscope (Axioskop). Alternatively, protein expression was determined by Western blot using 15 μg of lysates obtained by boiling the cells in 1% SDS.

**RESULTS**

The fibroblastoid morphology of several of the Sna-transfected HT-29 M6 and MDCK cell clones used in this work is shown in Fig. 1. The main attribute of these cells is the failure to form compact colonies and a more scattered aspect. In HT-29 M6 clones transfected with the tet-off system, the epithelial phenotype was restored by addition of the tetracycline analogue doxycycline, which prevents expression of Sna (Fig. 1 and Ref. 9).
Lateral sections obtained from HT-29 M6 clones illustrate that Snail overexpression prevented the formation of the compact columnar epithelial-like monolayer characteristic of HT-29 M6 cells and reduced the thickness from 12–16 to 4–6 μm (Fig. 2, left panels). HT-29 M6 SNA1 clone, like the other Sna-expressing clones, showed a flattened, fibroblast-like phenotype with cells disposed occasionally on the top of others. No cell contacts were observed in these transfectants in contrast to control HT-29 M6 clones where tight junctions, adherens junctions, and desmosomes were seen (Fig. 2, right panels). These control clones also showed closer apposition of lateral membranes and other characteristics of well differentiated epithelial cells like microvilli and mucus droplets.

Some cell properties were also altered in the Sna transfectants, compatible with the transition from an epithelial to a mesenchymal phenotype. In addition to a decrease in intercellular adhesion (Figs. 1 and 2, and data not shown), Sna-expressing clones attached better to plastic and spread faster on several matrices such as collagen or laminin (data not shown). Increases in these two parameters are observed in fibroblasts with respect to well differentiated epithelial cells like HT-29 M6. HT-29 M6 Sna clones treated with doxycycline were indistinguishable from controls (not shown).

We also determined whether these changes in phenotype were accompanied by alterations in the expression of different genes. Using a semiquantitative RT-PCR analysis, we found RNA levels of several epithelial genes, other than E-cadherin, to be decreased in Sna-transfected clones of HT-29 M6 cells (Fig. 3). Expression of MUC1 and Cytokeratin 18, which contain putative Snail-binding sequences in their promoters (see below), decreased in Sna-transfected clones. On the other hand, Fibronectin, LEF1, and ZEB1 were up-regulated in Sna transfectants. Increased levels of Fibronectin have already been reported in MDCK cells transfected with Snail (10); the augmentation in the synthesis of this protein might be responsible for the higher rate of attachment and spreading of Sna clones. ZEB1 and LEF1 are two transcription factors related to mesenchymal phenotypes. LEF1 is the preferential LEF/T cell factor isoform in mesenchymal cells and is differentially expressed in some neoplastic processes such as colon cancers (20). On the other hand, ZEB1 is a transcriptional factor recently described to be involved in E-cadherin repression (21). To investigate the changes in gene expression that accompany this epithelial to mesenchymal transition we decided to analyze in greater detail the modulation of two representative genes: one, MUC1, that is repressed, and another one, ZEB1, that is activated.
MUC1 is a marker of several epithelial tissues including the colonic epithelium. Its promoter sequence contains a tandem repeat of the consensus Snail DNA binding sequence situated 30 bp from the transcription start (22). Therefore, like Snail, it is a putative direct target of Snail. North–ern blots corroborated that MUC1 RNA levels were significantly lower in Sna-expressing HT-29 M6 clones than in controls (Fig. 4A). When an active ILK was expressed, IEC-18 cells transfected with empty vector; ILK (KD), IEC-18 cells transfected with a kinase-dead form of ILK; ILK (AS), IEC-18 cells transfected with an antisense cDNA of ILK; ILK-13, IEC-18 cells transfected with an active form of ILK.

The induction of ZEB1 expression was also investigated. As mentioned above, ZEB1 is also a transcriptional repressor capable, like Snail, of preventing the activity of the E-cadherin promoter. First, the expression of these three genes (SNA/Sna, ZEB1, and E-cadherin) was analyzed as a collection of epithelial cell lines expressing variable amounts of E-cadherin. Because of the low amounts of transcription factor RNAs present in a total RNA preparation, we used RT-PCR to detect Sna and ZEB1. Only in those cell lines in which Snail and ZEB1 were coexpressed was E-cadherin severely down-regulated (Fig. 5, upper panels). In a few cell lines, like EpH4, we detected simultaneous expression of both E-cadherin and Snail but not ZEB1.

We also analyzed whether the expression of Snail is induced in conditions in which cells undergo EMT. We used as a model IEC-18 epithelial cells that undergo EMT when transfected with ILK (14). When an active ILK was expressed, IEC-18 cells acquired a fibroblastoid phenotype that correlated with a down-regulation of E-cadherin. Although Snail was detected in these cells, overexpression of ILK induced an increase in the RNA corresponding to this gene. We found that the active ILK
form also induced ZEB1 RNA (Fig. 5, lower panels). Therefore, ZEB1 seems to be induced concomitantly with Snail and the acquisition of the fibroblastoid phenotype in IEC-18 cells.

The involvement of Snail in ZEB1 activation was evidenced by our data with Sna transfectants. As mentioned above (see Fig. 3), in our Sna-expressing HT-29 M6 clones ZEB1 RNA levels were increased. A more detailed study was performed in these clones taking advantage of the regulation of the expression of this gene by the tetracycline analogue doxycycline. ZEB1 RNA transcription was detected only after Snail RNA expression was turned on by withdrawing doxycycline from the medium (Fig. 6). Expression of Sna was detected 4 days after withdrawing doxycycline, slightly preceding ZEB1 expression, which required 8 days. Therefore, Sna expression is sufficient to trigger the ZEB1 RNA up-regulation. On the other hand, when Snail expression was turned off by adding doxycycline, ZEB1 RNA did not disappear immediately, as did Sna, but diminished gradually. The stability of ZEB1 RNA in the absence of Snail suggests that ZEB1 could possibly prolong the repression of epithelial genes initiated by Snail.

This result seems not to be exclusive of these HT-29 M6 clones since MDCK Sna-transfected clones also showed increased levels of ZEB1 RNA (Fig. 7A). The molecular mechanism by which Snail induces ZEB1 was studied. To test whether Snail induces the transcription of ZEB1, we cloned a 308-bp DNA fragment upstream of the ZEB1 first exon and assayed its promoter activity in MDCK control and Snaexpressing cells. Sna-expressing MDCK clones contained 2.5-fold higher activity than controls of this promoter (Fig. 7B), indicating that Snail increases ZEB1 RNA at least in part by the activation of the transcription of this gene. To validate this result we tested the reporter activity in transient transfection of Snail. In RWP-1 cells Snail also increased the transcriptional activity of the ZEB1 reporter; however, this effect was only observed when the activation was determined in cells transfected longer than 48 h (Fig. 7C).

The ZEB1 homologue, ZEB2, has also been described to behave as an E-cadherin repressor (23). However, it did not respond to Snail activation in any of our experiments, and its expression did not correlate with that of Snail in the epithelial cell lines tested; it was detected in all the cell lines analyzed (Figs. 5 and 7A).

As the first step to confirm the putative role of ZEB1 as a repressor in the Snail activated pathway, we confirmed that ZEB1 protein was also up-regulated in Sna-transfected clones. A clear increase of this protein was detected in the nucleus in HT-29 M6 and MDCK clones (Fig. 8). We also analyzed the repression activity of ZEB1 on E-cadherin promoter transcription in several epithelial cell lines using a proximal E-cadherin promoter (−178 to +23) upstream of a luciferase gene reporter. This promoter sequence contains three 5′-CACCTG-3′ boxes where Snail and ZEB1 can bind. ZEB1 repressed the promoter activity in a dose-dependent manner with a maximal inhibition of 60–80% in the cell lines tested, which included RWP-1 (Fig. 9A), MCF-7 (Fig. 9B), HT-29 M6, and MDCK (data not shown). The degree of repression obtained with ZEB1 and Snail were comparable; however, although both were expressed under the same promoter, ZEB1 required a 10–20-fold higher dose to achieve the maximum repression in the four cell lines assayed. This effect on the E-cadherin promoter was accompanied by changes in the expression of this protein (not shown). Thus, ZEB1 also holds the potential to repress E-cadherin transcription, although less effectively than Snail. On the other hand, ZEB2 repressed the promoter poorly with a maximum of 40% in MCF-7 cells (Fig. 9B). No synergistic effects were observed in experiments of simultaneous repression of Sna and ZEB1 on E-cadherin promoter; inhibitions of the activity of this promoter by both factors are additive (Fig. 9A).

We also tested whether ZEB1 repressed another Snail target gene, MUC1. Reporter assays using the above-described MUC1 promoter show that, in a fashion similar to Snail, ZEB1 repressed the promoter activity (Fig. 9C). Similar to the results obtained with E-cadherin promoter, ZEB1 was at least 10-fold less potent than Snail as a repressor of the MUC1 promoter.

**DISCUSSION**

In certain circumstances epithelial cells can undergo extensive changes in their phenotype and convert to mesenchymal cells. This EMT is observed in several phases of embryonic development and in carcinoma cell invasion and metastasis. Transcription of E-cadherin gene is down-regulated during these processes (4, 5). However, EMT involves a decrease of a set of epithelial tissue-specific molecules or markers other than E-cadherin and an increase of mesenchyme-related molecules (1). We (9) and others (10) have reported that the transcriptional repressor Snail blocks E-cadherin expression by binding to specific E-boxes in its promoter, but the mechanism by which levels of other molecules are regulated in EMT is poorly understood. Genetic approaches in Drosophila have shown that two transcription factors, Snail and Twist, regulate the expression of mesenchymal and epithelial genes, although some mesenchymal genes can also be regulated by other transcriptional genes like Dorsal and Tailless (11). Developmental studies (for review, see Ref. 24) as well as data obtained after transfection to epithelial cells indicate that Sna induces a complete EMT when transfected to cultured epithelial cells. We took advantage of this capacity of Sna to study the mechanisms of repression of epithelial genes or activation of mesenchymal genes.

Epithelial genes other than E-cadherin, like MUC1 and Cytokeratin 18, also possess E-boxes on their promoters. Our results show that RNA levels of these genes are decreased after Snail expression (Figs. 3 and 4) and that repression of MUC1 requires intact E-boxes (Fig. 4). Therefore, MUC1 and E-cadherin seem to be regulated by a common mechanism that involves Snail. However, other factors with affinity for this sequence may also participate in the down-regulation of MUC1 and E-cadherin transcription.

Among the genes up-regulated by Snail we found a transcriptional repressor, ZEB1, also capable of binding the same E-boxes as Snail. ZEB1 and ZEB2 are homologues of Drosophila Zfh-1, which is active downstream of Snail in embry-
Snail is required for the expression of Zfh-1 in the mesoderm primordium; in Snail mutants, Zfh-1 expression is severely reduced (25). Our results obtained from Sna-inducible and stable clones clearly show that Snail increases ZEB1 RNA (Figs. 5–7) and protein levels (Fig. 8) during EMT. We have also shown, through reporter experiments, that ZEB1 augmentation is due, at least in part, to an elevation of the transcriptional activity of the ZEB1 promoter (Fig. 9B). Although the ZEB1 promoter contains E-box sequences, we do not think that Snail directly activates ZEB1 promoter since (a) the time required to observe the increase of the reporter is longer than the time required to repress E-cadherin or MUC1 promoters (compare Figs. 7C and 9) and (b) ZEB1 expression was detected after 4 days of expression of Snail in a Sna-inducible clone (Fig. 6B).

ZEB1, like Snail, represses E-cadherin and MUC1 transcription when transfected into epithelial cells. Moreover, tumor cell lines with severe reduction of E-cadherin expressed both Snail and ZEB1 (Fig. 3), and Snail and ZEB1 repression activities were additive (Fig. 9). In addition to the up-regulation of ZEB1 promoter

**FIG. 7.** Snail induces ZEB1 promoter activity. A, RT-PCR analysis of Sna, ZEB1, and ZEB2 RNA in Sna-expressing MDCK clones. Canine ZEB1 and ZEB2 RNAs were amplified using oligonucleotides obtained from human ZEB sequences. B, activity of the ZEB1 promoter in MDCK control, SNA1, and SNA3 clones. Cells were transfected with pGL3 or pGL3-ZEB1 promoter for 48 h. C, activity of the ZEB1 promoter in RWP-1 transiently transfected with Sna. Cells were cotransfected with 100 ng of pGL3-ZEB1 promoter, 5 ng of pRLSV-Luc, and 10 or 50 ng of pRSV-Sna. Luciferase activity was assayed 3 or 6 days after transfection (d3 and d6, respectively). Results show the average ± range of two to three experiments performed in triplicate.

**FIG. 8.** Nuclear localization of Snail-induced ZEB1. HT-29 M6 (control and SNA1 clone) and MDCK (control and SNA1 clone) cells were stained with polyclonal antibodies against Sna (1:10 dilution) or ZEB1 (1:50 dilution) as described under “Materials and Methods.” Expression was determined in a fluorescent microscope. CONT, control.
RNA in Snail-induced clones, when Snail expression was switched off, ZEB1 expression required more than 20 days to gradually return to basal levels (Fig. 6). This observation indicates that in circumstances of a transient expression of Snail, ZEB1 prolonged Snail-induced repression of epithelial genes. In agreement with this hypothesis, in Drosophila embryos Zfh-1 persists after Snail is down-regulated (26, 27). However, whereas Sna knock-out mutant mice are not viable due to defective gastrulation (12), ZEB1 is dispensable for this process. ZEB1 knock-out animals develop to term, although they show severe deficiencies in T cell production in the thymus and several skeletal defects (28). Therefore, Snail, but not ZEB1, is necessary for the E-cadherin down-regulation required for a correct gastrulation.

The analysis of tumor cell lines with different epithelial or mesenchymal characteristics as well as IEC-18 intestinal cells expressing ILK showed a high inverse relationship between ZEB1 and E-cadherin expression. This relationship is better than that presented by E-cadherin and Snail since we detected several cell lines expressing these two proteins. However, recent data from our laboratory indicates that the presence of Snail does not always correlate with the activity of this factor since in several cell lines, in those that present concomitant expression of E-cadherin, Snail protein is excluded from the nucleus. These data, together with those obtained with knock-out mice, suggest that Snail is the key element controlling E-cadherin transcription and triggering EMT and, therefore, ZEB1 expression in most of epithelial cell lines. However, in some tumors, ZEB1 might become unresponsive to Snail activation as a result of mutations in the promoter or the activation of some of its transcriptional activators and might block E-cadherin expression.

All cell lines tested showed expression of ZEB2 independently of Snail and E-cadherin levels. This expression of ZEB2 was not modified either during ILK-induced EMT of IEC-18 cells or after induction of Snail. These observations strongly suggest a different regulation for the expression of the two ZEB homologues: ZEB1 is induced by Snail and is specific to fibroblastic cells, whereas ZEB2 is constitutively expressed in all the cell lines, independently of their epithelial or mesenchymal lineage. Moreover, although ZEB2 induces repression of E-cadherin promoter in some cellular contexts (Ref. 23 and Fig. 9B), this factor presents much lower activity than ZEB1 or Snail. The difference in the repression of E-cadherin between ZEB1 and ZEB2 may be due to the distinct organization of the regulatory domains (29). In any case, our results indicate that the endogenous amount of ZEB2 cannot repress the activity of basal epithelial promoters in tumor cells. However, due to its ubiquitous expression, the question of whether ZEB2 cooperates with Snail to repress epithelial promoters remains to be answered.

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