The transcription factor Snail downregulates the tight junction components independently of E-cadherin downregulation

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Accepted 21 November 2003
Journal of Cell Science 117, 1675-1685 Published by The Company of Biologists 2004
doi:10.1242/jcs.01004

Summary

Snail, a transcriptional repressor of E-cadherin expression, is involved in epithelial-mesenchymal transitions during development. We demonstrate that Snail activity is not restricted to E-cadherin downregulation. Expression of tight junction proteins, including claudin-1, occludin and ZO-1, was downregulated in MDCK cells exogenously expressing Snail protein. Although occludin mRNA levels were downregulated by Snail expression, the transcription of claudin-1 and ZO-1 were unaffected. Reporter assays using the claudin-1 promoter region revealed that promoter activity was not affected by Snail overexpression. Decreased synthesis of claudin-1 protein was observed, however, suggesting that Snail may act in translation initiation. Snail expression also altered the splicing pattern of p120. The levels of mRNA encoding the epithelial variant decreased, while the fibroblastic mRNA form increased. Although ectopic E-cadherin expression resulted in a downregulation of Snail-induced fibronectin expression, fibroblastic morphology was affected only minimally; the expression of tight junctional proteins remained at low levels. These results indicate that Snail is involved in both the direct transcriptional repression of genes, such as E-cadherin and occludin, and post-transcriptional events, including downregulation of claudin-1. These data support the idea that Snail is a transcription factor possessing pleiotropic activities.

Key words: Snail, Cadherin, Adherens junction, Tight junction

Introduction

Epithelial cells form cell-cell junctional complexes through tight junctions, adherens junctions and desmosomes. These junctions maintain epithelium structural integrity, creating a critical assembly for the physiological function of epithelial tissues. These junctional structures share a common organization, containing transmembrane adhesive components bound to cytosolic adapter proteins providing a link to the cytoskeleton. In adherens junctions, catenins link transmembrane E-cadherin to the actin cytoskeleton (Jou et al., 1995; Ozawa et al., 1990; Rimm et al., 1995). The transmembrane protein occludin in tight junctions is linked to the actin cytoskeleton by binding to ZO-1, which in turn binds to actin and ZO-2 (Furuse et al., 1994; Gumbiner et al., 1991; Ito et al., 1997; Stevenson et al., 1986). Desmosomal cadherins, distantly related to E-cadherin, are linked to intermediate filaments through cytosolic desmosomal plaque proteins, such as desmoplakin and plakoglobin (Buxton and Magee, 1992; Green and Jones, 1996).

During junctional complex assembly, cadherin-catenin complexes initially formed at sites of cell-cell contact (Adams et al., 1996; Angres et al., 1996) develop into mature adherens junctions. The cadherin superfamily of adhesion molecules possesses the common structural features of an extracellular domain with calcium-binding motifs, a single transmembrane domain and a C-terminal cytoplasmic domain. Classical cadherins, including E-, P- and N-cadherin, bind homotypically to cadherins neighboring cells (Nose et al., 1988). The conserved cytoplasmic domain then interacts with the cytoskeleton to enhance cell-cell adhesion (Ozawa et al., 1989; Ozawa et al., 1990; Reynolds et al., 1994).

The coordinated assembly of junctional complexes is dependent on E-cadherin (Gumbiner et al., 1988; Pasder and Nelson, 1988a). E-cadherin appears to reside at the top of the epithelial junctional complex assembly hierarchy. Incubation of epithelial cells in either low calcium concentrations or E-cadherin-blocking antibodies prevents proper assembly of adherens junctions, tight junctions and desmosomes (Gumbiner et al., 1988; Pasder and Nelson, 1988a; Pasdar and Nelson, 1988b; Siliciano et al., 1988). Furthermore, cells deficient in α-catenin also lack E-cadherin function, leading to defects in junctional complex assembly (Watabe et al., 1994). Mutant cadherins possessing truncated extracellular domains perturb cadherin-based adhesion and the formation of both adherens junctions and desmosomes (Amagai et al., 1995; Troxell et al., 1999; Troxell et al., 2000; Zhu and Watt, 1996). Thus, proper functioning of classical cadherins is necessary for desmosome assembly under these experimental conditions.

Snail is a zinc finger-containing transcription factor required for proper development of vertebrate and invertebrate embryos (Gray et al., 1994; Manzanares et al., 2001). This molecule triggers epithelial to mesenchymal transitions allowing epithelial cells to emigrate from their place of origin and form tissues such as the mesoderm and neural crest (Hay, 1995;

Duband et al., 1995). Snail also functions in epithelial to mesenchymal transitions responsible for the acquisition of invasiveness during tumor progression (Cano et al., 2000; Batlle et al., 2000; Blanco et al., 2002). This tumorigenic activity is associated with the ability of Snail to repress E-cadherin transcription (Cano et al., 2000; Batlle et al., 2000). Transfection of Snail cDNA into MDCK cells induced epithelial to mesenchymal transitions; transfected cells acquired motile and invasive properties concurrent with transcriptional downregulation of E-cadherin. While the morphological changes induced by Snail expression suggested that adherens junctions, tight junctions and desmosomes are all disrupted by Snail expression, the mechanisms controlling these changes have not been addressed comprehensively. In this study, we demonstrate that ectopic Snail expression downregulates the expression of E-cadherin and the expression of the tight junction components, occludin, claudin-1 and ZO-1. Occludin downregulation occurred at the transcriptional level in a manner similar to that of E-cadherin. The downregulation of claudin-1 and ZO-1, however, is not regulated at the transcriptional level. Introduction of an E-cadherin expression vector, which is not subject to Snail-mediated suppression, restored E-cadherin expression at the cell surface. Re-expression of E-cadherin, however, is not sufficient to re-establish tight junction protein expression and assembly. This result is consistent with the observation that Snail does not act indirectly through E-cadherin downregulation. We also demonstrate that Snail expression altered the p120 splicing variant expression pattern from the dominant epithelial form to the dominant fibroblastic form in MDCK cells.

Materials and Methods

cDNA construction

The cDNA encoding human Snail was isolated from a human heart cDNA library by polymerase chain reaction using the combination of specific sense (5′-CGGGATCCACTATGCCGCGCTCTTT) and antisense (5′-ATCGCGGGGACATCCTGAGCA) primers. Polymerase chain reaction was performed using *Pwo* DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). The PCR product was assembled into the pBluescript II KS (+) vector with the addition of a sequence encoding the nine amino acid hemagglutinin (HA) epitope. After confirming the sequence of the C-terminally HA-tagged human Snail cDNA, this fragment was cloned into the mammalian expression vector, pCAGGSneo.

The sequence encoding the HA tag within the HA-tagged mouse E-cadherin cDNA (Ozawa, 2002) was replaced by a sequence encoding a myc tag, to yield a myc-tagged E-cadherin construct. The cDNA was then cloned into the mammalian expression vectors, pCAGGSneo and pCAGGSpuro. The latter vector was constructed by replacing the neo gene with a puro gene, which encodes *Streptomyces alboniger* puromycin-*N*-acetyltransferase gene conferring resistance to puromycin.

Cells and transfection

MDCK cells (kindly provided by Y. Daikuhara, Kagoshima University Dental School) were grown in DME supplemented with 10% FCS. MDCK T23 cells, which express the tetracycline-repressible transactivator (Barth et al., 1997), and the pHMR272 plasmid, which carries the hygromycin resistance gene (Gossen and Bujard, 1992), were kindly provided by Dr W. J. Nelson (Stanford University). MDCK cells (5×10^5) were transfected with expression vectors (10 μg) by calcium phosphate methodology (Ozawa et al., 1989). G418-resistant clones (MDCK-Sna cells) were isolated as single colonies and examined for HA-tagged Snail expression by immunoblot and immunofluorescence analysis. MDCK-Sna cells were co-transfected with the mouse E-cadherin construct (10 μg) and pMIKHygB (1 μg) using LipofectAmine Plus (GibcoBRL, Gaithersburg, USA). Hygromycin B-resistant clones were isolated as single colonies and examined for myc-tagged E-cadherin expression by immunoblot and immunofluorescence analysis.

Antibodies

Mouse mAbs against E-cadherin, α-catenin, β-catenin, p120 (pp120) and fibronectin were purchased from Transduction Laboratories (Lexington, KY). A mouse mAb against vinculin was purchased from Sigma (St Louis, USA). Antibodies against claudin-1, claudin-3, claudin-4, claudin-7, occludin and Z0-1 were obtained from ZYMED (South San Francisco, USA). A mouse mAb recognizing Desmoplakins 1 and 2 was purchased from Progen Biotechnik GmbH (Heidelberg, Germany). A rat mAb against HA was purchased form Roche Diagnostics GmbH (Mannheim, Germany). A mAb specific for myc was obtained from Santa Cruz Biotechnology (CA, USA).

Immunoblotting and immunoprecipitation

For immunoblot analysis, cells were boiled for 5 minutes in SDS gel sample buffer (Laemmli, 1970). Proteins were separated by either 7% or 11% polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in PBS, then incubated with specific primary antibodies followed by treatment with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). After washing with PBS containing 0.1% Tween-20, protein bands were visualized by enhanced chemiluminescence (ECL) (Amersham International, Little Chalfont, UK). Immunoprecipitation was performed as described previously (Ozawa et al., 1989) with the following modifications. After washing in PBS, cells were lysed in 10 mM Tris-HCl buffer, pH 7.4, containing 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 0.1 mM sodium orthovanadate, 1 mM PMSF, 5 μg/ml leupeptin and 25 μg/ml aprotinin. The myc-tagged E-cadherin–catenin complex was collected using mouse anti-myc antibodies, preabsorbed to protein A-Sepharose CL4B (Amersham Pharmacia Biotech, NJ). Immunocomplexes were then washed in lysis buffer three times and boiled for 5 minutes in SDS-PAGE sample buffer.

RT-PCR Southern blot analysis

Poly $(A)^+$-RNA was extracted from cells using an mRNA Purification Kit (Amersham Pharmacia Biotech). RNA was reverse transcribed using M-MLV reverse transcriptase (GibcoBRL, Gaithersburg, USA). The resulting products were employed as templates for specific PCR reactions using *Pwo* DNA polymerase (Roche Diagnostics, Mannheim, Germany). The conditions for PCR were optimized for each primer pair. The following primer combinations were used: for E-cadherin, sense (GACACCCGATTCAAAGTGGG), antisense (GTCTCTCTTCTGTCTTCTGAG); for p120, sense (GCTGGATTT-GTCTTTCTCAGC), antisense (CCATCATCTGAGGTCTCCAC); for claudin-1, sense (CCAACGCGGGGCTGCAGCT), antisense (GG TTGTTTTTCGGGGACAGGA); for occludin, sense (GATGACTT-CAGGCAGCCTCG), anti- sense (CTATGTTTTCTGTCTATCA-TAGTC); for ZO-1, sense (ATGAAGACACAGATACAGAAGG), antisense (GGTGACCAACAGCTGGCTG); for GAPDH, sense (ACCACAGTCCATGCCATCAC), antisense (TCCACCACCCTG-TTGCTGTA). PCR products were then subjected to electrophoresis on 1.5% agarose gels. The DNA fragments were then capillary transferred to nylon membranes, followed by UV cross-linkage. All

of the probes used in Southern hybridization were labeled using the Gene Image random prime labeling module (Amersham Pharmacia Biotech). Probes were hybridized to the membranes during a 60°C overnight incubation. After hybridization, blots were first washed in 1×SSC, 0.1% SDS at 60°C for 15 minutes, then washed again in 0.1×SSC, 0.1% SDS under the same conditions.

Trypsin treatment and biotinylation of cell surface proteins

Trypsin treatment of cell surface proteins was performed as described (Ozawa and Kemler, 1990). To biotinylate cell surface proteins, 4.5×10^5 cells were seeded on 0.4 μm polycarbonate membranes (Nunc, Roskilde, Denmark) and cultured overnight. Following washing at 4°C in PBS (+), cells were soaked in 0.5 mg/ml Sulfo-NHS-Biotin (Pierce, Rockford, IS). Cells were then washed three times in PBS (+) and lysed in the immunoprecipitation buffer defined above. Immunoprecipitation of biotinylated proteins was performed as above using SoftLink™ Avidin Resin (Promega, Madison, WI). The remaining proteins were precipitated from the post-nuclear supernatants by ethanol precipitation. Isolated proteins and the ethanol-precipitated proteins were boiled for 5 minutes in SDS gel sample buffer and subjected to 7% SDS-PAGE. Samples were then transferred to nitrocellulose membranes and immunoblotted using an anti-E-cadherin antibody.

Reporter assay

The promoters of human E-cadherin (–178 to +66), occludin (–190 to +341) and claudin-1 (–82 to +236) were isolated by PCR from the genomic DNA of A431 cells using following combinations of specific primers for E-cadherin (5′-ACTCCAGGCTAGAGGGTCA and 5′-TGGAGCGGGCTGGAGTCT), occludin (5′-CTGGAGCTCTAGA-TGCCTTTTTCCAGCAA and 5′-GCGCTCTGGACCTGGCTC) and claudin-1 (5′-GGGCGCTCCCCGGCTG and 5′-GAAGATCTGA-CTCGCTCGGGCGCCC). After confirming the sequences, these fragments were cloned into pGL3-Basic vector (Promega). 2×10^5 A431 cells were seeded in 24-well plates 24 hours prior to transfection. One μg of reporter vector, 1 μg of pCAGGS-Snail and 20 ng of pRL were transfected per well using LipofectAmine Plus. After 48 hours, both firefly and Renilla luciferase activity was measured using the Dual Luciferase Reporter Assay Kit (Promega). Firefly luciferase activities were normalized to the Renilla luciferase activities.

Pulse-chase analysis

7×10^5 MDCK-neo and MDCK-Sna cells were plated on 6-well dishes. After an overnight culture, cells were labeled with 70 μCi/ml of [^{35}S] methionine in methionine- and cysteine-free DMEM supplemented with 10% dialyzed FCS for 16 hours. When specified, cells were labeled for 1, 3 or 6 hours. After culture in chase media for the indicated periods, cells were washed with ice cold PBS and lysed with RIPA buffer (1% TritonX-100, 0.5% sodium deoxycholate, 0.2% SDS, 150 mM NaCl, 2 mM EDTA, 10 mM HEPES (pH 7.4), 1 mM PMSF, 5 μg/ml leupeptin and 25 μg/ml aprotinin). Claudin-1 was collected with protein A-Sepharose beads conjugated to anti-claudin-1 antibodies, separated by gel electrophoresis and subjected to autoradiography.

Results

Expression of Snail in MDCK cells results in downregulation of tight junction components

Although the ability of Snail to induce epithelial to mesenchymal transitions and downregulate E-cadherin transcription has been well established, the molecular mechanisms underlying cellular morphological changes are poorly understood. To examine the effects of Snail expression on the levels of adherens junction and tight junction components, we introduced a hemagglutinin (HA) epitope-tagged human Snail cDNA into the MDCK epithelial cell line, to obtain Snail-expressing stable cell lines (MDCK-Sna cells) (Figs 1 and 2, HA). As reported previously (Cano et al., 2000; Batlle et al., 2000), MDCK-Sna cells exhibited epithelial-mesenchymal transitions, while control neomycin resistance cells (MDCK-neo) did not exhibit any morphological changes (Fig. 2, Phase). Immunofluorescence staining and immunoblot analysis with anti-E-cadherin antibodies revealed the loss of E-cadherin from cell-cell contact sites (Fig. 2, E-cad) and the overall downregulation of E-cadherin protein levels (Fig. 1, E-cad), respectively. RT-PCR/Southern blot analysis revealed only low levels of E-cadherin mRNA present in MDCK-Sna cells (Fig. 3). Despite the E-cadherin downregulation, approximately 50% of the β-catenin protein of MDCK-neo cells was detectable in MDCK-Sna cells (Fig. 1, β-catenin). Low levels of β-catenin were still detectable at cell-cell contact

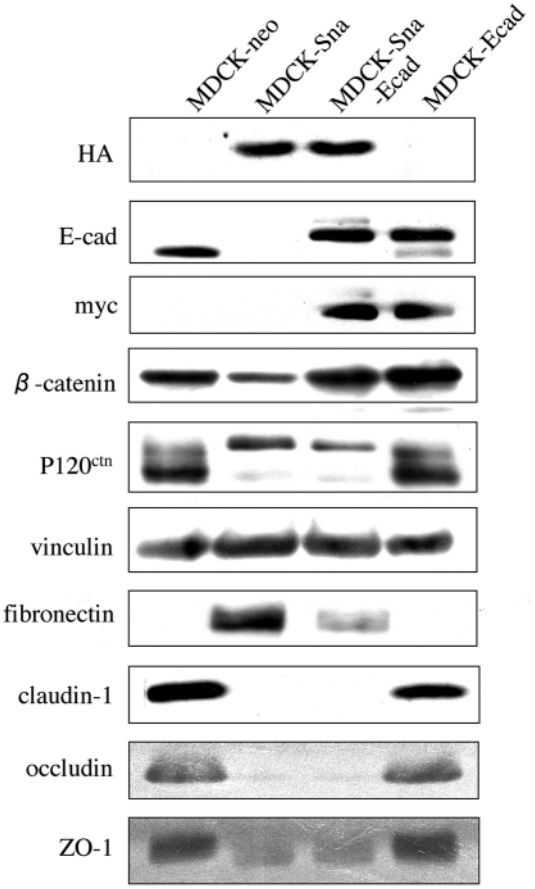

Fig. 1. Immunoblot analysis of the changes in cell junction protein expression. MDCK cell lines expressing control neo gene (MDCK-neo), Snail (MDCK-Sna), E-cadherin (MDCK-Ecad), or Snail and E-cadherin together (MDCK-Sna-Ecad) were lysed in SDS sample buffer and subjected to immunoblot analysis. HA-tagged Snail protein and ectopically expressed myc-tagged mouse E-cadherin protein were detected with the indicated antibodies. Myc-tagged E-cadherin migrates more slowly than the endogenous canine E-cadherin. Vinculin serves as an internal control for protein loading.

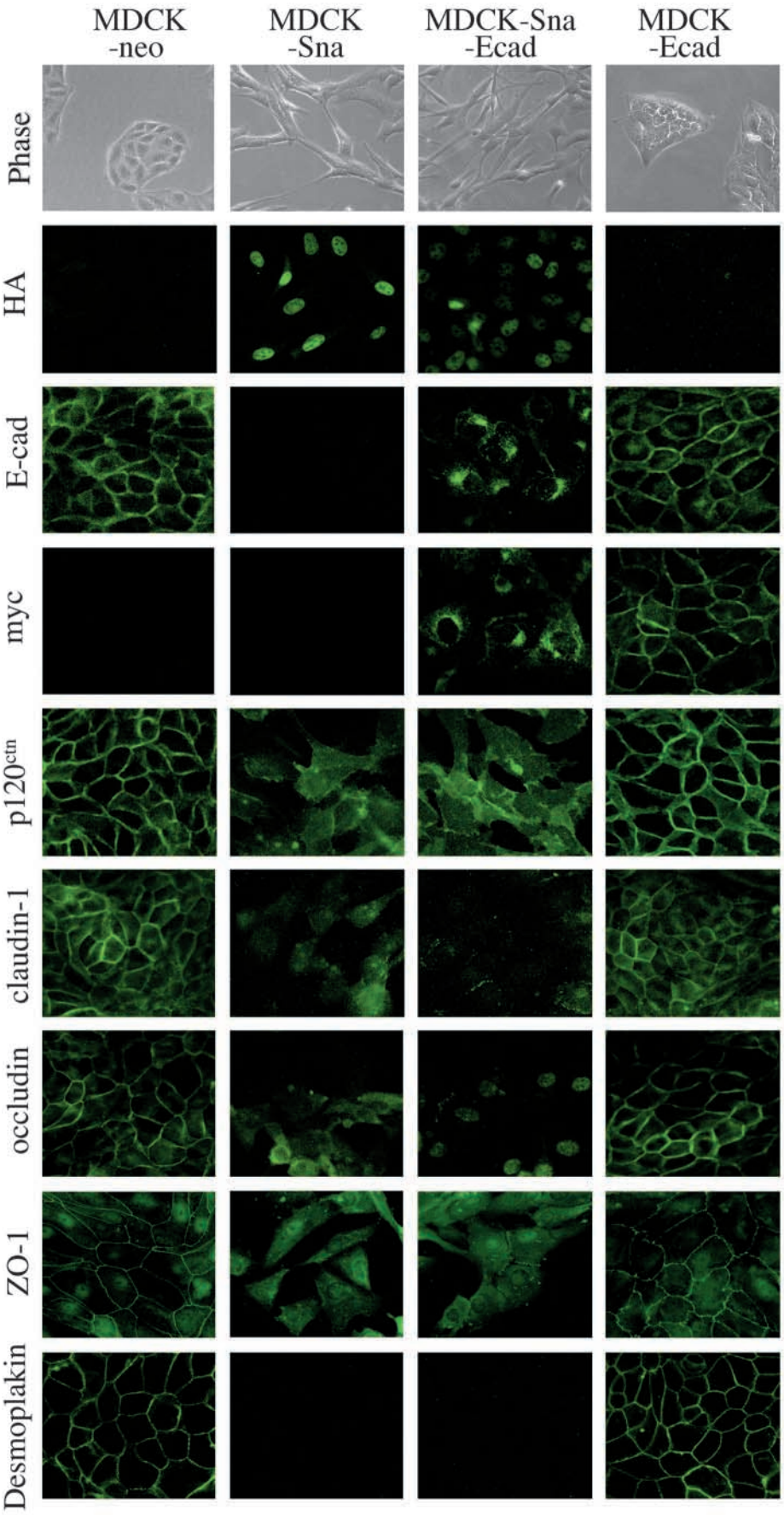

Fig. 2. Immunolocalization of cell junctional proteins. Proteins were detected with the indicated antibodies. Endogenous and ectopically expressed E-cadherin was detected using an anti-E-cadherin antibody. Only ectopically expressed E-cadherin was detected with an anti-myc antibody. Whereas no cell contact staining pattern of tight junctional proteins could be observed in MDCK-Sna cells, the additional ectopic expression of E-cadherin restored a minimal cell contact staining pattern.

sites (data not shown). Increased expression of additional cadherins recognizable by anti-pancadherin antibodies (data not shown) explains this observation.

The morphological changes and cell scattering induced by ectopic Snail expression in MDCK cells suggested the possible disruption of tight junction and desmosome complexes. To examine this hypothesis, cells were stained with antibodies specific for components of these junctional complexes. Staining specific for occludin, claudin-1, ZO-1 or desmoplakin could not be detected at the surface of MDCK-Sna cells (Fig. 2). Immunoblot analysis revealed that negative staining of cells for claudin-1 and occludin is due to their absence (Fig. 3B). A decreased amount of ZO-1 was detected in MDCK-Sna cells; this protein migrated faster than ZO-1 from MDCK-neo cells on SDS-PAGE (Fig. 3B). To determine the level at which Snail exerts an effect, we performed RT-PCR/Southern blot analyses. Consistent with previous reports (Cano et al., 2000; Batlle et al., 2000), the levels of mRNA encoding E-cadherin decreased significantly following ectopic Snail expression, whereas those for glyceralde-3-phosphate dehydrogenase were unaltered (Fig. 3). Occludin mRNA levels also decreased in MDCK-Sna cells, consistent with a recent report (Ikenouchi et al., 2003). In contrast, mRNAs encoding claudin-1 and ZO-1 were detectable in MDCK-Sna cells at levels similar to those seen in control MDCK-neo cells (Fig. 3). Thus, while ectopic Snail expression downregulates the transcription of E-cadherin and occludin, transcription of additional tight junction proteins was not significantly altered. Therefore, the major mechanism downregulating the expression of these proteins must be post-transcriptional.

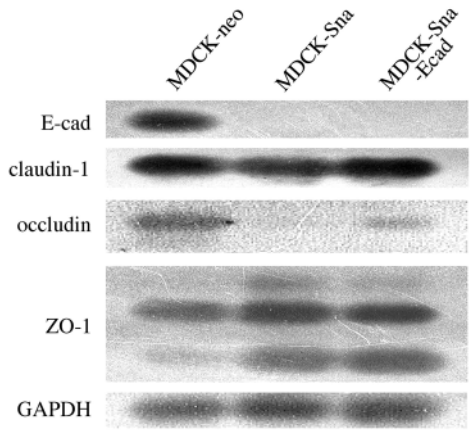

Fig. 3. Whereas the tight junctional protein occludin was transcriptionally downregulated following Snail expression, claudin-1 and ZO-1 were not. The mRNA expression levels of junctional proteins were detected by RT-PCR/Southern blot analysis using the indicated probes. While the expression of the α(+) ZO-1 isoform was not changed in each cell, the α(–) isoform expression levels were elevated in two Snail-expressing cells.

We carried out reporter assays to determine whether the downregulation of tight junction proteins occurs at the transcriptional level. As no sequence data is available for canine E-cadherin, occludin and claudin-1, we isolated the promoters for human occludin, claudin-1 and E-cadherin from the genomic DNA of A431 cells. Stable expression of Snail in A431 cells also results in the downregulation of E-cadherin, occludin, claudin-1 and ZO-1 (Fig. 4A). We transfected reporter constructs containing these promoters (Fig. 4B) into A431 cells and analyzed for promoter activity. Consistent with the RT-PCR analysis, the promoter activity of occludin was repressed by Snail expression in a manner similar to that of E-cadherin, whereas that of claudin-1 was not (Fig. 4C).

To determine the post-transcriptional events responsible for claudin-1 downregulation, we performed pulse-chase experiments. Cells were labeled with [^{35}S] methionine for 16 hours and then cultured in normal chase medium. Claudin-1 was immunoprecipitated with specific antibodies, run on gels and subjected to autoradiography. The amount of labeled claudin-1 obtained from MDCK-neo cells was much larger than that of claudin-1 from MDCK-Sna cells (Fig. 5). Labeling of cells for shorter periods (1, 3 and 6 hours) gave similar results (not shown). Thus, the synthesis of claudin-1 was decreased in MDCK-Sna cells in comparison with MDCK-neo cells.

Expression of Snail in MDCK cells results in changes in alternative splicing of p120 and ZO-1

Immunoblot analysis with anti-p120 antibodies revealed that MDCK-Sna cells express a species of p120 with an altered mobility from that in MDCK-neo cells (Fig. 1) or parental MDCK cells (not shown). MDCK-neo cells express proteins of 118 kDa and 100 kDa, with the latter appearing more prominently. In MDCK-Sna cells, the 120-kDa species was more prominent than the minor 100 kDa band. Following isolation by immunoprecipitation, phosphatase treatment of p120 protein increased their electrophoretic mobility slightly, as previously described (Ohkubo and Ozawa, 1999), but did not change the ratio of the intensities of the two forms. Thus, differential phosphorylation of p120 is not responsible for the altered electrophoretic mobility of p120 in MDCK-Sna cells. We observed the same electrophoretic mobility changes after ectopic Snail expression in A431 cells (data not shown), suggesting this is not a cell-type specific phenomenon. RT-PCR/Southern blot analysis revealed the presence of at least two p120 mRNA species produced by alternative splicing (Fig. 6). Direct sequencing of the PCR products identified an alternatively spliced sequence of 407 bp, corresponding to a published alternatively spliced nucleotide sequence. p120 mRNA isolated

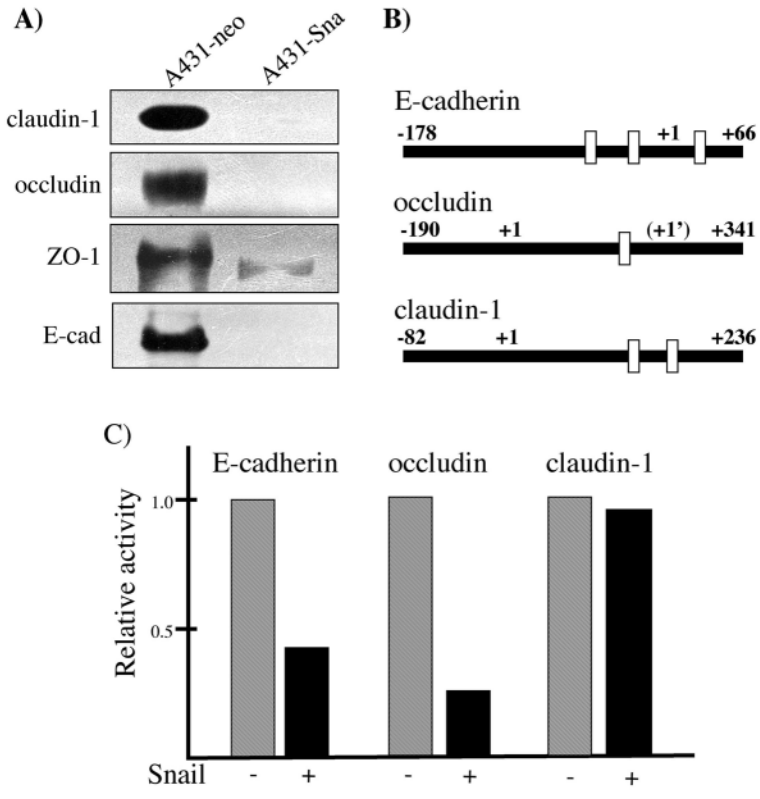

Fig. 4. Snail-induced downregulation of occludin expression, but not claudin-1 expression, is ascribed to suppression of promoter activity. (A) Downregulation by Snail of junction protein expression in A431 cells. (B) The promoter region of human E-cadherin, occludin and claudin-1. E-boxes are indicated by open boxes. The putative transcription start point is denoted by +1. For occludin, two putative transcription start points are proposed (Mankertz et al., 2000). (C) Repression of the promoter activities of human E-cadherin and occludin, but not that of claudin-1, by Snail in A431 cells. Reporter constructs carrying the human E-cadherin, occludin or claudin-1 promoters were co-transfected with a pCAGGS-Snail vector or an empty pCAGGS vector. Whereas the promoter activities of E-cadherin and occludin were repressed by Snail, that of claudin-1 was not repressed. The results represent the mean±s.d. of two independent experiments.

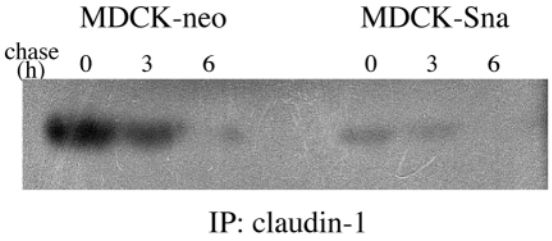

Fig. 5. Pulse-chase analysis of claudin-1 in MDCK cells. Cells were metabolically labeled with [^{35}S] methionine for 16 hours and chased for the indicated periods. Claudin-1 was collected by anti-claudin-1 immunoprecipitation; collected proteins were subjected to gel electrophoresis and autoradiography.

from MDCK-Sna cells contained the sequence absent from MDCK-neo cells (Fig. 6). Therefore, the p120 proteins detected by immunoblot analysis are the protein products of the alternatively spliced mRNA isoform.

RT-PCR/Southern blot analysis revealed the presence of at least three isoforms of ZO-1 in MDCK cells (Fig. 3). Sequencing (data not shown) revealed that the intermediate band represented a ZO-1 α(+) isoform (Willott et al., 1992), the conventional epithelial ZO-1 isoform (Sheth et al., 1997). The higher mobility band encoded the ZO-1 α(–) isoform. The ZO-1 α(–) isoform influences the plasticity of tight junctions (Balda and Anderson, 1993) and affects the early stages of membrane assembly (Sheth et al., 1997). Expression of this isoform increased in MDCK-Sna cells in comparison with MDCK-neo cells (Fig. 4). The identity of the larger band is as yet unknown.

Ectopic expression of E-cadherin in Snail-expressing mesenchymal cells did not induce epithelial morphological changes

E-cadherin is essential for maintaining epithelial morphology and for the proper formation of epithelial cell junctions, including adherens junctions, tight junctions and desmosomes (Gumbiner et al., 1988; Pasder and Nelson, 1988a; Pasdar and Nelson, 1988b; Siliciano et al., 1988). The expression of Snail in MDCK cells results in the transcriptional downregulation of E-cadherin and occludin, followed by the downregulation of the tight junction component claudin-1 at the post-transcriptional level. The expression of catenins is regulated at the post-transcriptional level (Papkoff, 1997); the stability of catenins increases significantly following cadherin expression in a variety of cells. Therefore, we were interested in which of the changes induced by Snail expression can be reversed by exogenous expression of E-cadherin. We introduced a mouse E-cadherin cDNA construct, tagged with a myc epitope, under the control of β-actin promoter and cytomegalovirus enhancer into MDCK-Sna cells to establish several stable cell lines expressing a myc-tagged E-cadherin (MDCK-Sna-Ecad cells) (Figs 1 and 2). As a control, MDCK cells transfected with the E-cadherin construct alone were also isolated (MDCK-Ecad cells). MDCK-Sna-Ecad cells retained fibroblastic morphology, despite exogenous expression of E-cadherin (Fig. 2). While the levels of E-cadherin protein expressed on MDCK-Sna-Ecad cells are similar to those of MDCK-Ecad cells and MDCK-neo cells (Fig. 1A), none of the MDCK-Sna-Ecad clones exhibited epithelial morphology.

In MDCK-Ecad cells, the majority of E-cadherin was observed at cell junctions by immunofluorescence staining, indicating that the expressed construct is successfully transported to the cell surface (Fig. 2). In MDCK-Sna-Ecad cells, however, E-cadherin was found intracellularly, concentrated in the perinuclear region, in addition to residing at cell-cell contact sites (Fig. 2). To confirm the intracellular staining pattern of ectopically expressed E-cadherin molecules resulting from Snail activity, we transfected MDCK cells established for ectopic E-cadherin expression (MDCK-Ecad cells) with the Snail construct. The isolated stable transfectants also showed intracellular staining of E-cadherin (data not shown). Thus, transport of E-cadherin to the cell surface is partially inhibited by Snail expression.

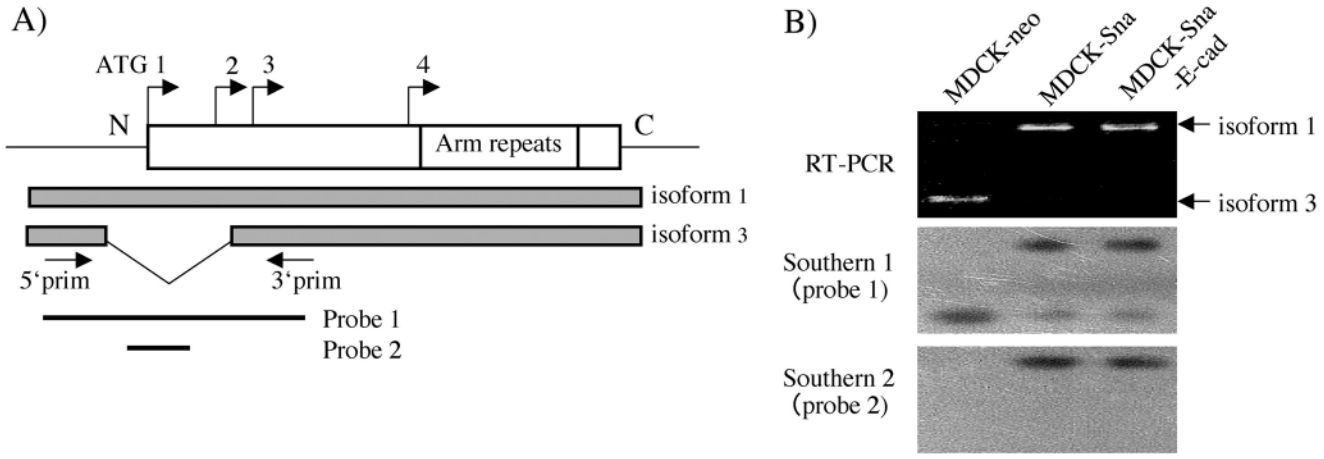

Fig. 6. RT-PCR/southern bolt analysis revealed changes in the relative p120 isoform levels in Snail-induced epithelial to mesenchymal transitions. (A) Schematic representation of four possible start sites, resulting from N-terminal splicing variations. The alternatively spliced 407 bp sequence is deleted from the N-terminus in isoform 3 cDNA. The primer positions for RT-PCR and the regions used to synthesize southern blot probes (probe 1, 2) are also depicted. (B) Using probe 1, N-terminally spliced shorter fragments were detected in MDCK-neo cells, while longer fragments derived from the fibroblastic isoform cDNA (isoform 1) were detected in MDCK-Sna and MDCK-Sna-Ecad cells by RT-PCR/Southern blot analysis. Only the longer fragments were detected using a splice region-specific probe (probe 2).

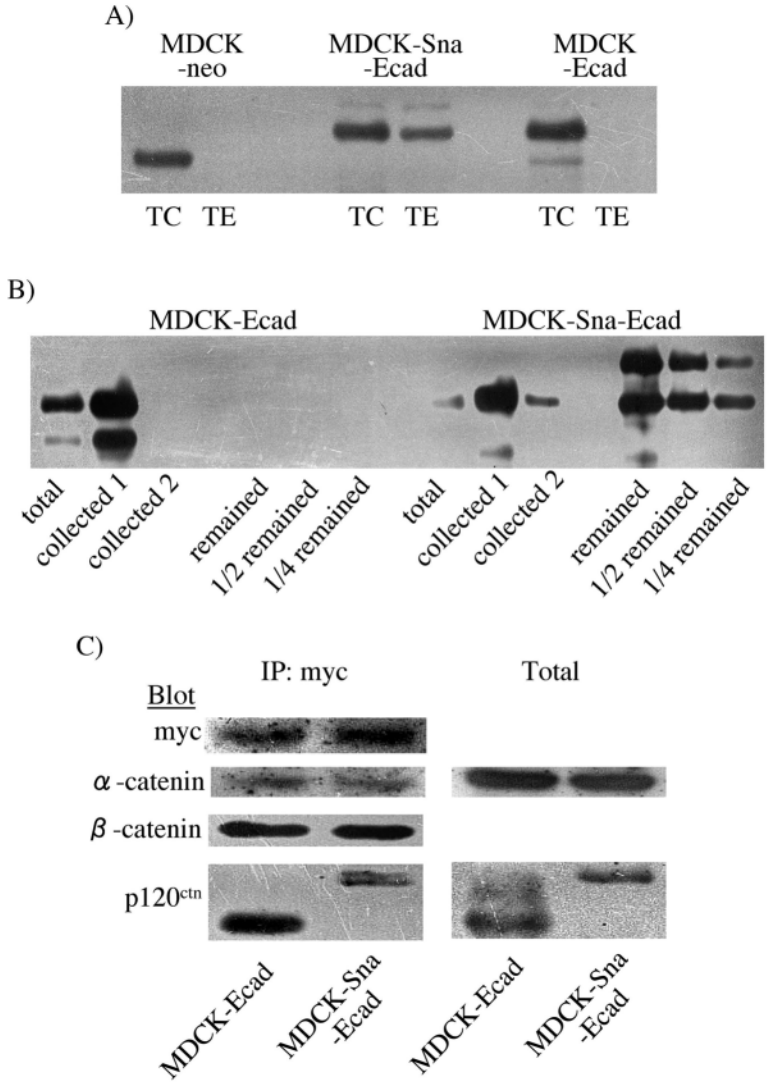

Fig. 7. Ectopic E-cadherin cell surface expression and association with catenins. The amount of surface E-cadherin was analyzed biochemically. (A) E-cadherin was detected by immunoblot analysis after trypsin/Ca^{2+} (TC) or trypsin/EGTA (TE) treatment. E-cadherin expressed on the cell surface was resistant to TC treatment, but sensitive to TE treatment. Only intracellular E-cadherin was detected following TE treatment. (B) E-cadherin was detected by immunoblot analysis after cell surface proteins were biotinylated and collected using avidin beads. After surface biotinylation, cells were lysed. After collection of biotinylated proteins (collected 1), the remaining lysate was re-collected (collected 2). The E-cadherin protein contained in either the collected (1) or remainder (2) portions were subjected to immunoblot analysis using an anti-E-cadherin antibody. To facilitate estimation, dilutions of the materials in each fraction were applied to the gels. (C) Formation of E-cadherin and catenin complexes in MDCK-Sna-Ecad cells. Cells were lysed and subjected to immunoprecipitation with an anti-myc antibody to precipitate ectopically expressed E-cadherin. Immunoprecipitates were subjected to immunoblot analysis using the indicated antibodies.

As a portion of the ectopically expressed E-cadherin remained in cytoplasm, we quantitated the amount of ectopic E-cadherin at the cell surface in MDCK-Sna-Ecad cells biochemically. Cell surface E-cadherins become resistant to trypsin digestion in the presence of Ca^{2+}, but can be degraded in the absence of Ca^{2+}. Thus, the pool of E-cadherin remaining undigested by trypsin in the presence of EGTA (TE treatment) represents the intracellular population; that remaining after trypsinization in the presence of Ca^{2+} (TC treatment) represents total cadherin. Immunoblot analysis of cells after TC and TE treatment using anti-E-cadherin antibodies revealed that all of the E-cadherin on control MDCK-neo cells and MDCK-Ecad cells was digested following TE treatment. Around 30-40% of the E-cadherin on MDCK-Sna-Ecad cells, however, remained undigested after TE treatment, indicating that these molecules reside intracellularly (Fig. 7A). Thus, only 60-70% of the total E-cadherin was expressed on the cell surface.

The cell surface levels of ectopically expressed E-cadherin are also supported by surface biotinylation studies. Cell surface proteins were biotinylated using sulfo-NHS-biotin; the biotinylated E-cadherin pool was collected using avidin beads (surface proteins). Non-biotinylated protein remained in the supernatant (intracellular pool). Both pools were examined by immunoblot analysis using anti-E-cadherin antibodies. Whereas almost all of E-cadherin in control MDCK-Ecad cells was biotinylated, only 40-50% of the E-cadherin in MDCK-Sna-Ecad cells was biotinylated (Fig. 7B). While these cell surface biotinylation studies detected a lower degree of E-cadherin on the cell surface, the discrepancy between these methods may be explained by incomplete biotinylation of surface proteins. The reason for an increased relative ratio of E-cadherin retaining the precursor segment in the intracellular pool relative to the total E-cadherin in the biotinylation experiments is unknown. We concluded that, in contrast to control cells, approximately 50% of the total E-cadherin was expressed on the cell surface of MDCK-Sna-Ecad, while the remainder remained localized intracellularly.

Ectopic expression of E-cadherin increased the levels of catenins, as measured by immunoblot analysis (Fig. 1). The amounts of catenins in MDCK-Sna-Ecad cells increased to the reach levels similar to those seen in epithelial MDCK-neo cells or MDCK-Ecad cells, suggesting that the ectopically expressed E-cadherin can associate with catenins. The shift in the expression pattern of p120 induced by Snail expression, characterized by the presence of the larger isoform, was not altered by ectopic E-cadherin expression (Figs 1 and 6). Immunoblot analysis of the ectopically expressed E-cadherin immunoprecipitates with anti-catenin antibodies revealed the association of ectopically expressed E-cadherin with catenins (Fig. 7C). α-Catenin and β-catenin co-precipitated with the ectopic E-cadherin to the same extent as seen in control MDCK-Ecad cells. The larger isoform of p120 also co-precipitated with ectopic E-cadherin in MDCK-Sna-Ecad cells.

Minimal staining of tight junctional components was observed in MDCK-Sna cells (Fig. 2). Ectopic expression of E-cadherin did not induce significant changes in the staining pattern or intensities of these components (Fig. 2). Faint junctional staining was occasionally observed in MDCK-Sna-

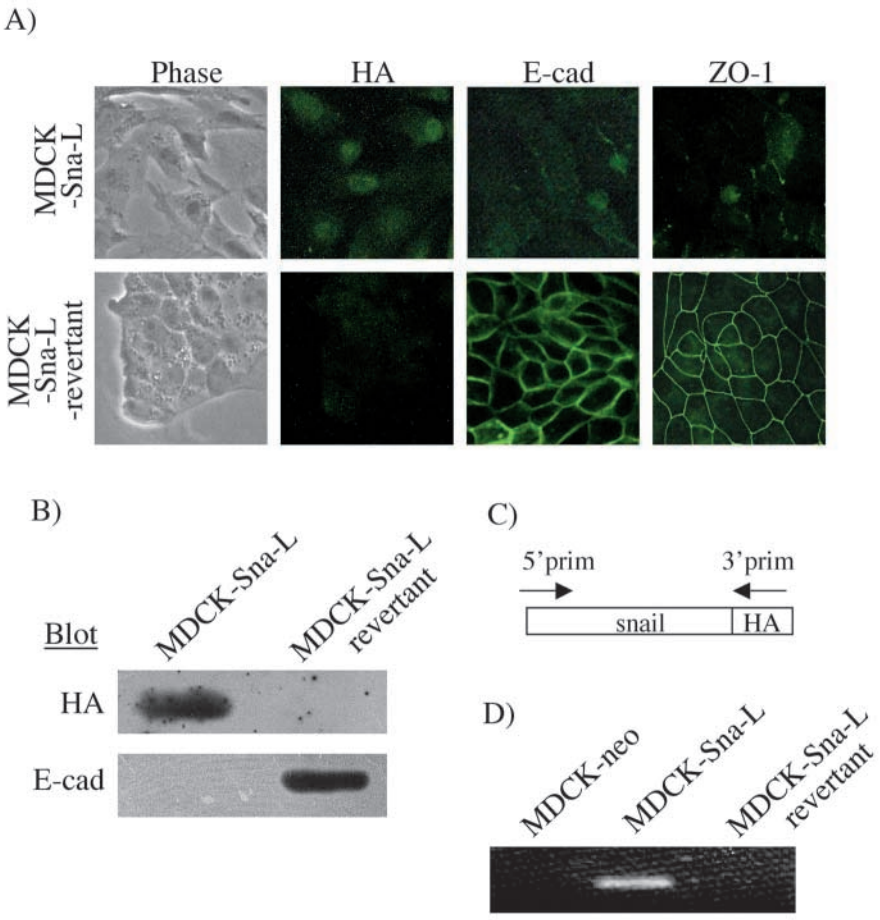

Fig. 8. Cessation of Snail expression reversed the mesenchymal phenotype into an epithelial phenotype. (A) MDCK cells expressing low levels of Snail (MDCK-Sna-L) and revertant cell lines lacking Snail expression were immunostained with the indicated antibodies. (B) Snail and E-cadherin expression were examined by immunoblotting. Owing to low expression levels, HA-tagged Snail proteins were collected with an anti-HA antibody and then subjected to immunoblot analysis. (C) The Snail-HA gene and the positions of the primers used to detect the transgene. The following specific primers were used. Snail 5′ primer, ACTATGCCGCGCTCTTTCCTC and HA 3′ primer, GTCGTAGGGGTA GCCGATATC. (D) Detection of the transgene. PCR was performed on genomic DNA isolated from the three A431 cell lines indicated. The transgene could not be detected in A431-Sna-L revertant cells.

Ecad cells. Regions staining faintly for occludin, claudin-1 and ZO-1 coincided with regions staining intensely for E-cadherin. The expression levels of these tight junctional proteins, however, were not altered by exogenous E-cadherin expression (Fig. 3B). Thus, the presence of E-cadherin is not sufficient for either the formation of tight junctions or the post-transcriptional regulation of these proteins. In addition, ectopic expression of occludin in MDCK-Sna cells did not induce any changes in the expression of tight junction proteins (data not shown).

Despite the lack of an apparent effect of exogenous E-cadherin expression on cell shape and tight junction, the presence of exogenous E-cadherin protein suppressed the Snail-induced expression of fibronectin (Fig. 1), supporting previous evidence demonstrating the downregulation of fibronectin expression by exogenous cadherin expression (Finnemann et al., 1995).

Cessation of Snail expression reversed the epithelial-mesenchymal transition in MDCK-Sna cells

Attempts to express Snail protein in MDCK cells under the control of the tetracycline-repressible transactivator were not successful for unknown reasons. Therefore, we attempted to isolate revertant cell lines. We had obtained several MDCK-Sna cell clones, all of which demonstrated mesenchymal morphology and downregulated E-cadherin expression, despite significant differences in Snail protein expression levels. A clone expressing relatively low amounts of Snail (MDCK-Sna-L) was subcloned twice by limiting dilution to eliminate possible contamination of Snail-negative cells with epithelial morphology. After several weeks in G418-free medium, epithelial cell islands appeared amongst the fibroblastic cells. These epithelial cells were re-established as cell lines and tested for Snail expression. The expression of Snail had been completely lost in these cell lines, as revealed by both immunofluorescence staining (Fig. 8A) and immunoblotting (Fig. 8B). To determine whether the cessation of Snail expression is due to the disappearance of the integrated snail-HA transgene from the genomic DNA of the revertant cells, we examined the isolated cells for the presence of the transgene. Genomic DNA was isolated from the cells and subjected to PCR analysis using primers specific for the transgene (Fig. 8C). The snail-HA transgene was detected in MDCK-Sna-L cells, but not in MDCK-Sna-L revertant cells (Fig. 8D). The complete junctional staining pattern of E-cadherin, ZO-1 (Fig. 8A), claudin-1 and occludin (not shown) was re-established in the revertant cell lines; levels of E-cadherin protein expression were also elevated (Fig. 8B). The reappearance of complete epithelial-like revertants from Snail-expressing mesenchymal cells indicated that ectopic Snail expression did not induce the differentiation of MDCK cells into mesenchymal cells, but instead induced morphological changes in the cells of our system.

Discussion

Epithelial to mesenchymal transitions are essential morphological conversions occurring during embryonic development, which also occur during metastatic cancer progression. During this mesenchymal transition, epithelial cells dissociate from each other, accompanied by the disruption of epithelial junctional complexes. As E-cadherin is a major

constituent of adherens junctions that probably plays an essential role in the assembly of junctional complexes and the maintenance of epithelial cell morphology, functional downregulation of E-cadherin correlates well with epithelial to mesenchymal transitions. The epithelial to mesenchymal transition of mouse mammary epithelial cells requires E-cadherin downregulation and the upregulation of β-catenin transcriptional activity (Stockinger et al., 2001). The transformation of mammary epithelial cells into mesenchymal cells following the overexpression of integrin-linked kinase also involves the downregulation of E-cadherin at both the protein and mRNA levels. (Somasiri et al., 2001).

The transcriptional repressor, Snail, is expressed in regions undergoing epithelial to mesenchymal transitions during development, such as the early mesoderm and neural crest (Nieto, 2002). This molecule effectively downregulates E-cadherin transcription (Cano et al., 2000; Batlle et al., 2000). In addition, several malignant tumors demonstrate an inverse correlation between Snail and E-cadherin expression (Cano et al., 2000; Batlle et al., 2000; Blanco et al., 2002). Snail expression induces drastic morphological changes in MDCK cells, causing these cells to lose the integrity of their cell-cell contacts. These results suggest that Snail disrupts the function of adherens junctions as well as other cell-cell junctions, including tight junctions. Our results confirm this hypothesis, demonstrating that disorganization of tight junction results from a lack of junctional components. As occludin mRNA decreased significantly, occludin downregulation occurs at the transcription level, consistent with recently published studies (Ikenouchi et al., 2003). The downregulation of claudin-1, however, appears to be post-transcriptional; we could detect claudin-1 mRNA in Snail-expressing MDCK cells. A lack of repression in reporter assays by Snail of human claudin-1 promoter activity, which is also downregulated by Snail expression in A431 cells, supports this conclusion. Pulse-chase analysis observed the decreased synthesis of claudin-1 in Snail-expressing cells in comparison with control neo-MDCK cells. Further studies will be necessary to determine the mechanism by which Snail, a transcriptional repressor, reduces translation of claudin-1. Snail blocks E-cadherin transcription by binding to specific DNA sequences (CANNTG, where N is any nucleotide), called E-boxes, within the promoters of target genes (Cano et al., 2000; Batlle et al., 2000). The human occludin promoter contains one E-box (Mankertz et al., 2000), while the mouse and human claudin-1 promoters each have two E-boxes (Kramer et al., 2000). Thus, the presence of the E-box DNA sequences within the promoter is not sufficient to be suppressed by Snail. Recently, Snail was shown to suppress the promoter activity of claudin-3, claudin-4 and claudin-7 (Ikenouchi et al., 2003). Consistent with this, we observed downregulation of these claudins in Snail-expressing MDCK cells (Ohkubo, T., unpublished observation). While we do not know the reason for the differential suppression of claudin promoter activities by Snail, we hypothesize that there must be functional differences between these claudins allowing these differences.

Downregulation of ZO-1 is unexpected, as ZO-1 is expressed ubiquitously in a variety of cell types, including fibroblasts (Itoh et al., 1993). The decreased expression of ZO-1 upon TGF-β-induced transdifferentiation of mammary epithelial cells to mesenchymal cells has been reported (Miettinen et al., 1994). Interestingly, the levels of mRNA encoding an alternative splice variant of ZO-1, the α(–) isoform, were increased following Snail expression. These levels could not be altered by ectopic E-cadherin expression. This isoform may influence the plasticity of tight junctions (Balda and Anderson, 1993) or may function in the early stages of membrane assembly (Sheth et al., 1997).

Analysis of the multiple alternatively spliced isoforms of p120 present in a variety of murine cell lines indicates that motile cells, such as fibroblasts and macrophages, preferentially express isoform 1, while epithelial cells preferentially express isoform 3 (Mo and Reynolds 1996; Keirsebilck et al., 1998). Isoform 1 expression is dramatically upregulated in a Src-transformed MDCK cell line, indicating that the pattern of isoform expression can be altered following cellular transformation. Isoform switching did not strictly correlate with transformation, however, as not all Src-transformed cell lines exhibited this effect (Mo and Reynolds, 1996). Contrary to this observation, all MDCK-Sna and MDCK-Sna-Ecad clones demonstrated isoform switching. This is the first report that Snail expression alters the splicing pattern of p120.

We also observed an unexpected retardation of E-cadherin transport to the plasma membrane in cells overexpressing Snail. Ectopically expressed E-cadherin remained primarily within the Golgi apparatus (T.O., unpublished observation). One possible explanation for this localization is that junctional complex maturation at cell contact sites was disturbed by Snail expression, either directly or indirectly. This disturbance may signal to the trans-Golgi network to halt the supply of additional E-cadherin molecules to sites of cell contacts. The possibility remains, however, that Snail expression may also regulate the trans-Golgi network system directly, possibly through interactions with a rab GTPase-dependent vesicle transport system. Further studies will be necessary to resolve this question.

Overexpression of integrin-linked kinases in mammary epithelial cells, which may stimulate Snail expression, transforms these cells into mesenchymal cells (Somasiri et al., 2001; Tan et al., 2001). While the type of transition induced by integrin-linked kinase overexpression can be reversed by forced expression of E-cadherin (Somasiri et al., 2001), Snail-induced epithelial to mesenchymal transitions could not be reversed by such ectopic expression. Detailed analysis revealed that while E-cadherin expression could reverse the upregulation of fibronectin expression, it could not reverse the downregulation of tight junction components. Although a portion of ectopically expressed E-cadherin was retained internally, biochemical data demonstrated that approximately 50% of the total E-cadherin was expressed at the cell surface. Recent analysis of MDCK cells expressing a mutant E-cadherin indicated that substantial desmosome and tight junction assembly occurred in cells overexpressing mutant E-cadherins, despite downregulation of endogenous E-cadherin and possible inhibition of the remaining E-cadherin by dominant-negative and/or competitive effects (Troxell et al., 1999; Troxell et al., 2000). Thus, low levels of cadherin function appear to be sufficient for cell-cell junctional complexes to assemble and remain stable. In that sense, our findings seem to be an apparent contradiction to previous studies. The action of Snail is thus not restricted to E-cadherin

downregulation, but is rather pleiotropic, additionally inducing the downregulation of tight junctional components. Thus, it is reasonable that the expression of E-cadherin cannot reverse the transition induced by Snail.

Catenin expression is regulated at the post-transcriptional level; association with cadherins stabilizes these proteins. In contrast, ectopic expression of occludin could not restore the expression and localization of tight junctional proteins. This result is consistent with the observation that the subcellular localization of claudin was not affected by the loss of occludin expression. In addition, tight junctions are not affected either morphologically or functionally in occludin-deficient mice (Saitou et al., 2000). Therefore, independent mechanism(s) are probably involved in the post-translational regulation of claudin-1 and ZO-1 levels.

The increased expression of an additional cadherin, detectable with pancadherin antibodies, may be responsible for the failure of ectopic E-cadherin expression to reverse some of the Snail-induced changes. Expression of N-cadherin in epithelial cells also induces epithelial to mesenchymal transition and increased motility (Kim et al., 2000).

A variety of intracellular signals regulate tight junction assembly and function (Madara, 1998), including the Rho family of GTPases. These small GTPases are actin cytoskeletal regulatory molecules controlling cortical actin structures and tight junction assembly in epithelial cells (Gopalakrishnan et al., 1998; Jou and Nelson, 1998; Jou et al., 1998; Nusrat et al., 1995; Takaishi et al., 1997). Rho-family GTPases localize to the apical junctional complex in epithelial cells (Jou and Nelson, 1998; Jou et al., 1998; Takaishi et al., 1997). Future experiments will need to address the actin regulatory mechanisms disrupted by Snail expression and the effects of this disruption on tight junction function.

We thank Rolf Kemler, Yasushi Daikuhara, James Nelson, Akihiko Yoshimura and Ken-ichi Yamamura for providing us with reagents, Misako Haraguchi for her helpful advice with the reporter assays, and Kumiko Sato for secretarial assistance. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in Aid for Science Research on Priority Area (B).

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