Ectopic expression of Snail in MDBK cells does not induce epithelial–mesenchymal transition

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Abbreviations: EMT, epithelial–mesenchymal transition; MDBK, Madin-Darby bovine kidney; MDCK, Madin-Darby canine kidney.

Running title: Lack of EMT response in Snail-expressing MDBK cells.

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 expression induces EMT in Madin-Darby canine kidney (MDCK) cells and the human epidermoid carcinoma cell line A431. Snail is a zinc finger transcription factor and triggers EMT by repressing E-cadherin expression. To extend our knowledge of Snail-induced EMT, we generated stable Snail transfectants using Madin-Darby bovine kidney (MDBK) cells. Contrary to MDCK or A431 cells, MDBK cells transfected with the Snail construct maintained an epithelial morphology and showed no sign of reduced cell–cell adhesiveness relative to control cells. Consistent with these observations, downregulation of epithelial marker proteins, e.g., E-cadherin and desmoglein, and upregulation of mesenchymal marker proteins, e.g., N-cadherin and fibronectin, were not detected. Furthermore, the E-cadherin promoter was not methylated. Therefore, in MDBK cells ectopic Snail expression failed to induce EMT. Although in MDCK cells Snail expression is accompanied by the increased expression of other EMT-inducing transcription factors, e.g., Slug and ZEB1, Snail-MDBK cells did not exhibit increased expression of these factors. Thus, it is possible that the failure to upregulate other EMT-related transcription factors may have explained the lack of Snail-mediated EMT induction in MDBK cells.

1. Introduction

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 (1, 2). In addition to tissue remodeling, organ development, and wound healing, EMT plays a critical role in cancer progression (3-6). 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 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 the formation and maintenance of epithelial structure (7). Desmoglein is a desmosome component and is expressed in desmosome-bearing epithelial cells (8). E-cadherin and desmoglein are members of the cadherin family of cell–cell adhesion molecules.

A hallmark of EMT is the loss of E-cadherin expression (9). Several transcription factors, including Snail, Slug, Twist, and ZEB1, have been implicated in the transcriptional repression of E-cadherin and EMT induction (9, 10). Snail belongs to the Snail superfamily of zinc finger transcription factors (11). Snail and Slug, a related superfamily member, are expressed during development in the early mesoderm and neural crest (12-14). These two zinc finger transcription factors repress E-cadherin transcription through an interaction of their

C-terminal regions with a 5'-CACCTG-3' sequence (termed an E-box) in the cadherin promoter (15, 16). Correlative studies have shown that there is an inverse relationship between E-cadherin expression and Snail expression in human samples (17).

The EMT is accompanied by epigenetic modifications, including DNA methylation (18, 19). DNA methylation, which is commonly associated with gene repression and heterochromatin formation, is defined by the addition of a methyl group to the cytosine of a CpG dinucleotide in a gene's promoter region (20). Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates a broad range of cellular responses (21). TGF-β is the major mediator of EMT and induces the expression of Snail (22) and Slug (23). Recent studies revealed that the effects of Snail on epithelial cells include promotion of the expression of other EMT-inducing transcriptional factors such as ZEB1 (24), and activation of the TGF-β signaling pathway (25). Cells exposed to TGF-β undergo EMT, which includes E-cadherin promoter DNA methylation (26, 27)

The ectopic expression of Snail in several epithelial cells, including Madin-Darby canine kidney (MDCK) cells and the human epidermoid carcinoma cell line A431, resulted in EMT (28, 29). The precise molecular events that initiate the complex EMT process, however, are poorly understood. To further understand the role of Snail in EMT, we generated stable Snail transfectants using a bovine cell line, Madin-Darby bovine kidney (MDBK) cells. Surprisingly, MDBK cells transfected with the Snail construct maintained their epithelial

morphology and showed no sign of reduced cell–cell adhesiveness relative to control cells. Consistent with these observations, downregulation of the epithelial marker proteins E-cadherin and desmoglein, and upregulation of the mesenchymal marker proteins N-cadherin and fibronectin were not detected. Furthermore, the E-cadherin promoter was not methylated. Therefore, in MDBK cells, ectopic Snail expression failed to induce EMT. Although Snail expression in MDCK cells is accompanied by the increased expression of other EMT-inducing transcription factors, such as Slug and ZEB1, MDBK cells ectopically expressing Snail did not show increased expression of these factors. Thus, it seemed that the inability to upregulate the expression of additional EMT-inducing transcription factors could explain the failure of ectopic Snail protein to induce EMT in MDBK cells.

Materials and methods

Cell lines and transfection

MDBK cells, a bovine kidney epithelial cell line provided by Dr. Rolf Kemler (Max-Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany), were grown and were transfected as previously described (28) using the calcium phosphate method with 10 μg of either plasmid DNA containing an HA-tagged human Snail construct (pC-SnailHA) or with control empty vector containing a neomycin resistance gene.

Antibodies

Mouse mAbs against E-cadherin, p120, and fibronectin were purchased from BD Biosciences (Lexington, KY). A mouse mAb against vimentin was obtained from Zymed Laboratories (South San Francisco, CA). Mouse mAbs recognizing Snail and Slug were purchased from Cell Signaling Technology (Danvers, MA). A mAb against desmoglein 1 + 2 was purchased from Progen Biotechnik GmbH (Heidelberg, Germany). A goat antibody recognizing ZEB1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse mAb recognizing vinculin was purchased from Sigma-Aldrich (St Louis, MO). A rat mAb against HA was purchased from Roche Applied Science (Mannheim, Germany). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

RT-PCR analysis

Total RNA was extracted and reverse transcribed as described previously (29). The resulting cDNA was used as a template for PCR and PCR conditions were optimized for each primer pair as previously described (29). The following primer combinations were used: E-cadherin, sense (5'-GACACCCGATTCAAAGTGCAC-3') and antisense (5'-GTCTCTCTTCTGTCTCCTGAG-3'); Slug, sense (5'-GCGTTCTCCAGACCCTGGT-3') and antisense (5'-GCACAGCAGCCAGACTCCT-3'); Twist1, sense (5'-GAGTCCGCAGTCCTACGAG-3') and antisense (5'-TCTGTAGGACCTGGTAGAGG-3'); ZEB1, sense

(5’-TGGGCAGTGACGGTAGGTAT-3’) and antisense (5’-GCAGGTCGAACCTCTTGATC-3’); β-actin, sense (5’-CAAGGACCTCTACGCCAACA-3’) and antisense (5’-CGTACTCCTGCTTGCTGATC-3’).

Cell aggregation assay

Cell aggregation assays were performed as previously described (30). In brief, cells were incubated for 10 min at 37 °C in HEPES-buffered saline containing 0.01% trypsin (type XI, Sigma-Aldrich) and 2 mM $CaCl_2$ or 1 mM EGTA. After the addition of soybean trypsin inhibitor (Sigma-Aldrich), the cells were washed, resuspended, and incubated for 30 min at 37 °C with constant rotation at 70 rpm. The extent of cell aggregation was represented by the index: (Nc−Np)/Nc, where Np and Nc were the total number of particles and cells per dish, respectively.

Immunoblotting

For immunoblot analysis, proteins were separated by 8% polyacrylamide gel electrophoresis, and were transferred to nitrocellulose membranes. After blocking membranes 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 previously described (31).

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 previously described (31). Cells were analyzed using an Olympus fluorescence microscope (Tokyo, Japan) or a confocal laser scanning microscope (LSM700; Zeiss).

DNA methylation analysis.

Genomic DNA (~0.75 μg) was treated with sodium bisulfite using the EpiTect system (Qiagen, Germantown, MD). Bisulfite-converted DNA (~400 ng) was used as a template for PCR amplification of the CpG islands in the CDH1 promoter. The primer pairs were sense (5'-GAGATTTGAAGTTTAAAAGATAGAA-3') and anti-sense (5'-AACTAAAATCTAACAAAACTTCTAC-3'). PCR products were purified from a 1.5% agarose gel using a Gel Extraction Kit (Qiagen) and cloned into the pGEM-T Easy vector (Promega). Four or five randomly selected clones from each sample were selected for sequencing. As a positive control for methylated DNA, genomic DNA was methylated in vitro using CpG methyltransferase (M.SssI; New England BioLabs Inc., Ipswich, MA).

Results

Ectopic Snail expression does not induce morphological changes or change the adhesiveness of MDBK cells.

MDBK cells, a cell line derived from bovine kidney, display epithelial properties, including brick stone morphology. We introduced a control empty vector containing a neomycin resistance gene or an expression vector encoding HA-tagged Snail protein into MDBK cells and isolated stable transfectants, designated neo or Snail, respectively. Snail cells retained the same epithelial morphology as control neo cells (Figure 1), despite clear nuclear localization of Snail protein as revealed by staining with an anti-HA antibody. Thus, contrary to previous experiments with MDCK or A431 cells (28, 29), ectopic expression of Snail did not induce morphological changes that were characteristic of EMT.

Cells undergoing EMT lose cell–cell adhesion. It is well known that cadherins at the cell surface resist tryptic digestion in the presence of Ca^{2+}, but not in the absence of Ca^{2+} (7). Therefore, cell aggregation assays after tryptic digestion of cells in the presence of either 2 mM Ca^{2+} or 1 mM EGTA can be used to distinguish between cadherin-mediated and cadherin-independent cell–cell adhesion. Cell aggregation assays demonstrated Ca^{2+}-dependent, cadherin-mediated cell–cell adhesion in both neo cells and Snail cells; no significant differences in cell–cell adhesion were observed between the two cell populations. These results are consistent with the morphological observation that Snail cells were not undergoing EMT.

Ectopic Snail expression in MDBK cells does not change the expression levels of epithelial and mesenchymal markers.

Next, we determined the expression levels of the epithelial markers, E-cadherin and desmoglein, using immunoblot analysis (Figure 2). Although Snail cells expressed exogenous Snail protein as detected by anti-HA antibodies, they showed essentially the same expression levels of E-cadherin and desmoglein as control neo cells. Control neo cells also expressed the mesenchymal markers, N-cadherin, vimentin, and fibronectin, and the expression levels of these proteins did not increase in Snail cells (Figure 2, Table 1). Thus, the ectopic expression of Snail in MDBK cells did not lead to the downregulation of E-cadherin or desmoglein expression or to the upregulation of N-cadherin, vimentin, or fibronectin expression. Furthermore, as previously reported (28), the expression of Snail altered the splicing patterns of p120 in MDCK cells, but not in MDBK cells (Figure 2).

Consistent with the observations that Snail expression did not change cadherin-mediated cell–cell adhesion (Figure 1) or the expression levels of E- or N-cadherin (Figure 2), immunofluorescence staining revealed that E- and N-cadherin were detected at the plasma membrane of both neo and Snail cells (Figure 2).

The E-cadherin promoter is not methylated in MDBK cells ectopically expressing Snail protein

Previous analysis of the E-cadherin gene revealed that its proximal promoter contains CpG islands, which are targets for methylation during TGF-β–induced EMT (26, 27). Therefore, we examined the methylation status of the E-cadherin promoter. No significant de novo DNA methylation was detected at the E-cadherin promoter in Snail cells as compared to control neo cells as measured by bisulfite sequencing (Figure 3). These results were consistent with the observation that no significant downregulation of E-cadherin expression was detected in Snail cells.

Ectopic expression of Snail protein in MDBK cells does not increase the production of EMT-related transcription factors

As previously reported, the expression of LEF-1, an EMT-inducer, in MDCK cells resulted in the significantly increased expression of other EMT-inducing transcription factors, including Slug and ZEB1 (31). Using an Agilent Whole Canine Genome microarray, we found that ectopic Snail expression in MDCK cells resulted in the increased expression of Slug and ZEB1 (Ozawa unpublished data). The upregulation of Twist and ZEB1 expression and the induction of EMT in human mammary epithelial HMLE cells upon Snail overexpression have been reported (32). Therefore, we used RT-PCR to compare the mRNA expression levels of Slug, Twist, and ZEB1 in neo cells and Snail cells. We observed no significant changes in the mRNA levels of these factors upon ectopic Snail expression (Figure 4). Furthermore, immunoblot analysis revealed that MDCK cells expressing Snail increased Slug and ZEB1 production at the

protein level, but that MDBK cells expressing Snail did not. Thus, our data suggest that Snail-mediated upregulation of Slug and ZEB1 is necessary to downregulate E-cadherin expression and induce EMT.

Discussion

In this study, we showed that the ectopic expression of Snail in MDBK, a bovine kidney epithelial cell line, failed to induce changes that were characteristic of EMT. None of the following events were observed: 1) epithelial to fibroblastic morphological changes; 2) reduced cell–cell adhesion; 3) downregulation of the epithelial markers E-cadherin and desmoglein; or 4) upregulation of the mesenchymal markers, N-cadherin, vimentin, and fibronectin. Although downregulation of E-cadherin and desmoglein in human squamous cell carcinoma HSC-4 cells is not extensive (33), transfection of cells with the Snail construct used in the present study induced EMT in a number of cell lines of different origin, including canine kidney epithelial MDCK cells (28, 29), the human epidermoid carcinoma cell line A431 (28, 29), the human squamous cell carcinoma HSC5 (34), and the murine embryonal carcinoma cell P19 (G. Izawa unpublished observation).

Exogenous Snail expression has been reported to suppress the activity of an E-cadherin promoter–reporter construct in MDCK cells, but not in mouse mammary epithelial NMuMG cells (35). In that study, the reason for the cell context–dependent Snail activity was

not analyzed. Snail protein undergoes posttranslational modifications, including GSK3β-mediated phosphorylation (36), and PDK1-mediated phosphorylation (37), followed by ubiquitination, which leads to Snail protein degradation. Although wild-type Snail protein could not induce EMT in MCF7 cells, mutant Snail protein—in which serine residues that are targets for GSK3β phosphorylation were substituted with alanine residues—was stabilized and did induce EMT (36). Therefore, the failure of Snail protein to induce EMT in MCF7 cells was explained by its rapid turnover rate and low protein expression in this cell line (36). Since the protein levels of Snail in MDBK cells were very similar/comparable (>70%) to those in MDCK cells, it seems less likely that rapid turnover and low protein levels were responsible for the failure of Snail protein to induce EMT in MDBK cells. Consistent with this idea, the addition of the GSK3β inhibitor BIO (6-Bromoindirubin-3'-oxime) did not induce EMT in MDBK cells ectopically expressing Snail (G. Izawa, unpublished observation). Phosphorylation regulates the subcellular localization of Snail protein (38). Immunostaining of Snail, however, revealed a significant portion of Snail is present in the nucleus (Fig. 1B).

The levels of EMT-inducing transcription factors are under the control of microRNAs, which are regulated by wild-type p53 (39, 40). Therefore, the presence of wild-type p53 has been proposed to be responsible for the failure of overexpressed Snail protein to induce EMT in MCF7 cells (32). MDBK cells seem to express wild-type p53 (41). Thus, the same mechanism could be operating in MDBK cells to suppress Snail activity. However,

MDCK cells, in which overexpressed Snail does induce EMT, also have wild-type p53 (42). Therefore, the presence of wild-type p53 alone cannot explain the failure of Snail to induce EMT in certain cell lines.

As previously reported, the expression of LEF-1, an EMT-inducer, in MDCK cells resulted in the significantly increased expression of other EMT-inducing transcription factors, e.g., Slug and ZEB1 (31). The upregulation of Twist and ZEB1 expression and the induction of EMT in HMLE cells upon Snail overexpression have also been reported (32). Therefore, the expression of multiple EMT-inducing factors seems to be necessary to complete the EMT process. As shown in the present study, ectopic Snail expression increased Slug and ZEB1 production at the protein level in MDCK cells, but not in MDBK cells. Double transfectants of MDBK cells expressing Snail and Slug showed no sign of EMT (Izawa unpublished observation). Thus, failure to upregulate multiple EMT-inducing factors may underlie the inability of ectopic Snail expression to induce EMT in MDBK cells.

It has been shown that shRNA-mediated knockdown of E-cadherin induces EMT (43). Thus, knockdown of E-cadherin expression seems to be an essential step for EMT induction. Although E-cadherin suppression during EMT is commonly associated with CpG island methylation within its promoter, our bisulfite sequencing analysis revealed that the E-cadherin promoter was not methylated in MDBK cells ectopically expressing Snail protein, and immunoblot analysis showed that E-cadherin expression was maintained in those cells.

Therefore, failure to downregulate E-cadherin expression may also explain why Snail-expressing MDBK cells did not undergo EMT.

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Figure Legends

Figure 1. MDBK cells ectopically expressing Snail protein display epithelial characteristics. (A) Both control MDBK cells transfected with an empty vector containing a neo-resistance gene (neo) and MDBK cells transfected with an expression vector encoding HA-tagged Snail protein (Snail) display typical epithelial cell morphology. (B) Immunofluorescence staining

with an anti-HA antibody shows the expressed Snail protein in the nucleus, which is co-stained with DAPI. (C) Cell aggregation assays show that cells ectopically expressing Snail protein have similar adhesive properties as the control (neo) cells; furthermore, the observed cell–cell adhesion is calcium-dependent, indicating that it is mediated by cadherins. Bars, 20 μm.

Figure 2. EMT is not induced in MDBK cells ectopically expressing Snail. (A) Immunoblot analysis reveals that Snail expression in MDBK cells does not decrease the expression of the epithelial markers E-cadherin and desmoglein and does not increase the expression of the mesenchymal markers N-cadherin, vimentin, and fibronectin. α-tubulin is used as a loading control. (B) The ectopic expression of Snail alters the splicing patterns of p120 in MDCK cells, but not in MDBK cells. (C) E-cadherin (E-cad) and N-cadherin (N-cad) are detected at the membrane of control (neo) and cells ectopically expressing Snail protein (Snail). Cells were stained with the appropriate primary antibody followed by a rhodamine-labeled secondary antibody. DAPI was used to detect the nucleus. Bar, 20 μm.

Figure 3. Ectopic expression of Snail in MDBK cells does not induce DNA methylation of the E-cadherin promoter. Diagram showing the position of four E-boxes (-403 to -398, -201 to -196, -151 to -146, and -100 to -95; red bars) and CpG dinucleotides within the E-cadherin

promoter region (circles). Genomic DNA was isolated from control (neo) and Snail cells, and methylation of the E-cadherin promoter was analyzed by bisulfite sequencing. Genomic DNA incubated with CpG methyltransfease prior to bisulfite treatment was used as a positive control for methylated DNA. Methylated and unmethylated dinucleotides are indicated as filled and open circles, respectively.

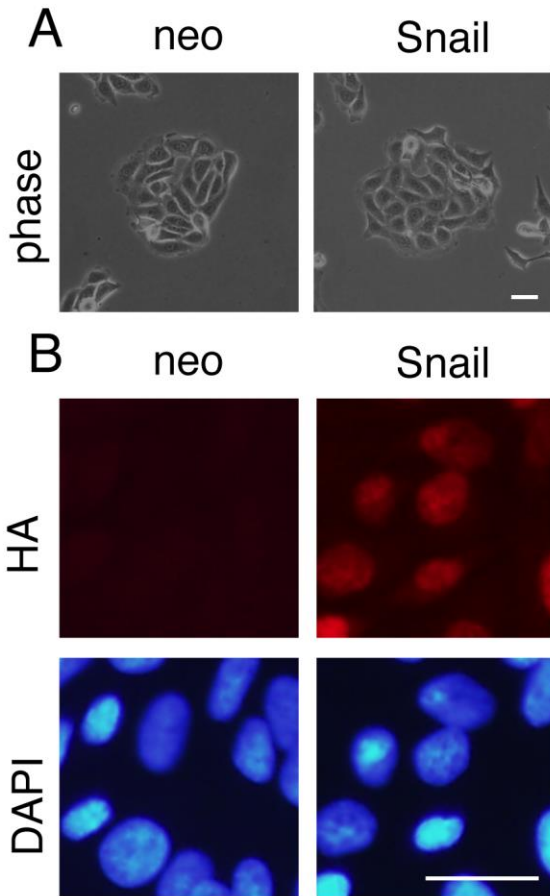
Figure 4. (A) RT-PCR analysis of Slug, Twist, and ZEB1 mRNA in control (neo) cells and Snail cells. β-actin is used as an internal control. No significant differences are observed between control cells and Snail cells with respect to the mRNA levels of these proteins. (B) Immunoblot analysis using anti-Slug and anti-ZEB1 antibodies. Vinculin serves as a loading control. Ectopic Snail expression increases Slug and ZEB1 protein levels in MDCK cells, but not in MDBK cells. Ectopic Snail expression in MDBK cells slightly increased the expression level of ZEB1 protein, but quantification of the blots using NIH Image revealed that the relative amounts of ZEB1 protein in Snail-MDBK cells were less than 20% of that in Snail-MDCK cells.

Table I. Relative expression levels of epithelial and mesenchymal markers in MDBK cells ectopically expressing Snail protein.

	E-cadherin	desmoglein	N-cadherin	fibronectin	vimentin
Ratios	0.76 ± 0.09	0.87 ± 0.06	0.74 ± 0.13	0.74 ± 0.13	1.16 ± 0.12

The expression levels were determined using ImageJ (National Institutes of Health). The data are presented as the relative intensity of the bands in Snail-MDBK cell samples as compared to control (neo) MDBK cell samples. Values are the mean ± S.E. obtained from three independent clones.

Fig. 1.



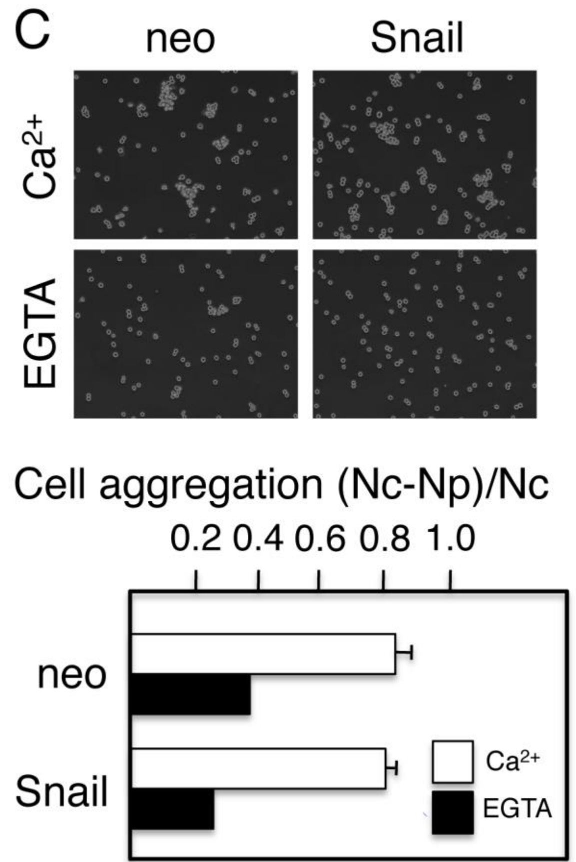


Fig. 1.

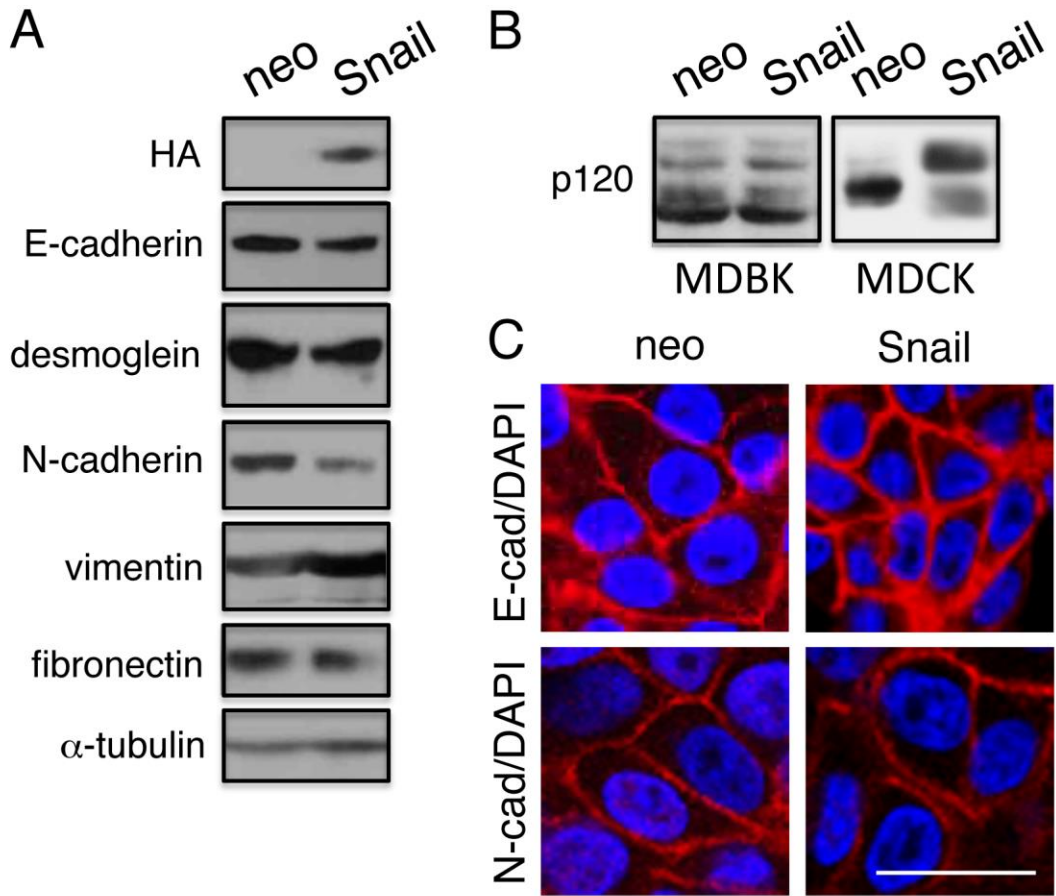


Fig. 3.

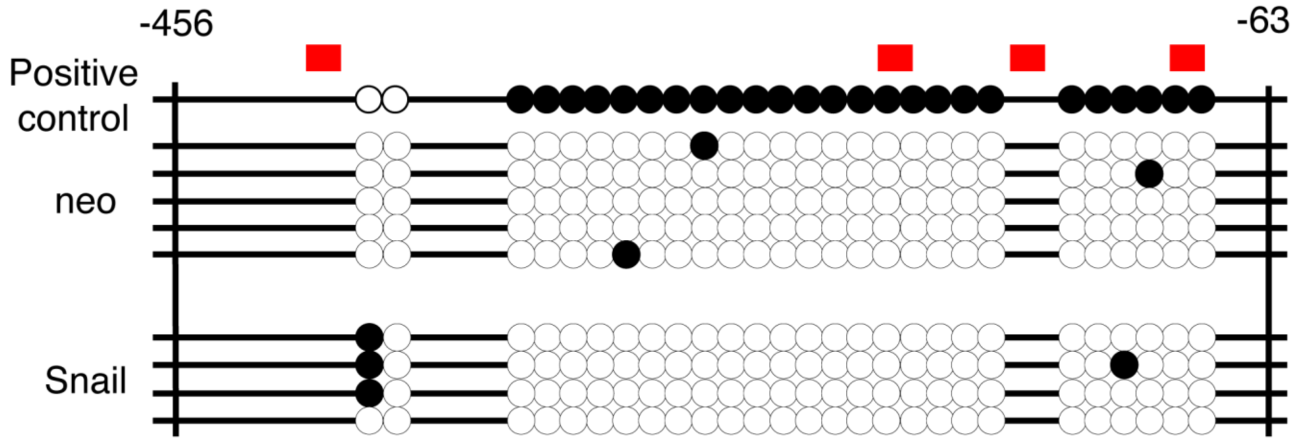


Fig. 4.