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Reversibility of the Snail-induced epithelial–mesenchymal transition revealed by the Cre–*loxP* system

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ABSTRACT

The 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. Snail is an EMT-inducer whose expression in several different epithelial cells, e.g., Madin–Darby canine kidney (MDCK), leads to EMT. To further understand EMT induced by Snail expression, the Cre–*loxP* site-specific recombination system was used to investigate its reversibility. Transfection of MDCK cells with loxP-flanked Snail (Snail-loxP) resulted in EMT induction, which included the acquisition of a spindle-shaped fibroblastic morphology, the downregulation of epithelial markers, and the upregulation of mesenchymal markers. DNA methylation of the E-cadherin promoter, which often occurs during E-cadherin downregulation, was not observed in Snail+ cells. After Cre-mediated excision of Snail-loxP, the cells reacquired an epithelial morphology, upregulated epithelial markers, and downregulated mesenchymal markers. Thus, EMT induced by Snail expression was reversible.

1. Introduction

The loss of epithelial characteristics and the gain of a mesenchymal phenotype, a process referred to as an epithelial-to-mesenchymal transition (EMT), is considered to be a hallmark of neoplasmic transformation [1]. A key initial step in EMT is the downregulation of E-cadherin, a cell–cell adhesion protein that is repressed at the transcriptional level by ZEB1, ZEB2, Snail, Slug, and Twist [2]. The loss of E-cadherin is accompanied by the upregulation of mesenchymal markers, such as N-cadherin. Concomitant with these molecular changes, cells acquire a spindle-shaped mesenchymal morphology and display enhanced migratory and invasive properties.

Snail is a transcriptional repressor that plays a central role in EMT. Snail can repress E-cadherin transcription through an interaction of the Snail C-terminal region with a 5′-CACCTG-3′ sequence (referred to as an E-box) in the cadherin promoter [3,4]. It has been shown that Snail bound to the E-cadherin promoter interacts with and recruits histone deacetylase (HDAC), which in turn induces the deacetylation of histones within the E-cadherin promoter, a feature associated with transcriptionally inactive chromatin [5]. EMT is accompanied by epigenetic modifications, including DNA methylation [6]. 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 [7].

Transforming growth factor-β (TGF-β) is the major mediator of EMT and induces the expression of Snail [8] and Slug [9]. Cells exposed to TGF-β undergo EMT, which includes methylation of the E-cadherin promoter [10,11]. Exposing mammary gland cells to TGF-β induces EMT, including the loss of E-cadherin; however, TGF-β withdrawal promotes a mesenchymal-to-epithelial transition (MET) and triggers the re-expression of E-cadherin, suggesting that TGF-β-induced EMT can be a reversible process [12]. By contrast, prolonged TGF-β treatment establishes a mesenchymal state that is stabilized by an autocrine TGF-β signaling network with ZEB [13]. It is assumed that cells in a stabilized EMT state also exhibit DNA methylation to repress epithelial gene expression. For example, the exposure of cells to TGF-β for 12 days induces maximal methylation of the E-cadherin promoter, and those cells do not reverse to the epithelial state upon withdraw of TGF-β treatment [11].

Abbreviations: EMT, epithelial–mesenchymal transition; MET, mesenchymal–epithelial transition.

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Importantly, methylation of the E-cadherin promoter in cells that have undergone a stabilized EMT remains unchanged, suggesting that methylation of the E-cadherin promoter is a relatively stable memory marker in cells undergoing long-term EMT [11]. Thus, mesenchymal cells become difficult to revert to the epithelial state once the E-cadherin promoter has been methylated.

Stable silencing of Snail expression by shRNA has been shown to derepress E-cadherin expression and lead to a MET in the stably transfected MDCK-Snail system [14]. Although the abrogation of Snail expression by the shRNA appears to be highly specific, possible off-target effects cannot be excluded, because rescue experiments with cDNA constructs that are resistant to silencing by the shRNAs were not performed.

The Cre recombinase of the P1 bacteriophage catalyzes recombination between two specific 34-bp consensus sequences (loxP sites) with high specificity and efficiency [15]. Introduction of the Cre enzyme will result in recombination between the loxP sites and excision of the intervening DNA sequence. Use of the Cre/loxP system in eukaryotic cells requires only that the components (the Cre enzyme and the loxP sites) be introduced [16]. Here, we show that expression of loxP-flanked Snail (Snail-loxP) in MDCK cells promotes EMT. After Cre-mediated excision of Snail-loxP, the cells reacquired an epithelial morphology, upregulated epithelial markers, and downregulated mesenchymal markers. Thus, EMT was reversed and MET had occurred.

2. Materials and methods

2.1. Plasmids

The Snail-loxP vector was constructed as follows. The HA-tagged human Snail (Snail-HA) coding sequence followed by a rabbit β-globin polyadenylation signal (pA) [17] was inserted between two tandem loxP sites. The loxP-flanked Snail-HA/pA was cloned into the XhoI site of the pCAGG vector [18], which contains the CMV enhancer/chicken β-actin promoter (CAG). A DNA fragment encoding a hygromycin resistance gene followed by the SV40 polyadenylation signal of the pSilencer™ 3.1-H1 hygro vector (Life Technologies Japan, Tokyo, Japan) was inserted downstream of the loxP-flanked Snail-HA/pA, to produce the Snail-HA-loxP vector (see Fig. 1A). The Cre expression plasmid, pCAG/NCre, containing the CAG promoter and the bacteriophage P1 Cre gene with DNA encoding a nuclear location signal (NLS) attached to its 5′ end [19], was provided by Masahiro Sato (Kagoshima University).

2.2. Cells and transfection

MDCK cells (5×10^6) were transfected with 10 μg of the Sal I–linearized expression vector, pC-SnailHAloxP, using electroporation with a Bio-Rad Gene Pulser (Bio-Rad Japan, Tokyo, Japan) set at 220 V and 960 μF. After selection with G418, single colonies were isolated and analyzed by immunofluorescence staining and immunoblot analysis using anti-HA antibodies. As a control, we used an empty vector containing a neo-resistance gene (neo). At least three independent clones were selected for each construct to ensure that any observed effects were not due to the phenotypic variability inherent to transfection. The experiments with these clones gave essentially the same results. Transfection of Snail-loxP MDCK cells with the pCAG/NCre plasmid was performed with the Amaxa nucleofector system (Amaxa GmbH, Cologne, Germany) as previously described [20]. Two days after transfection, cells were incubated with medium containing 5 μg/ml of puromycin (Clontech, Mountain View, CA, USA) for an additional 2 days to isolate drug-resistant colonies. Selecting the drug-resistant and the positive and negative HA-stained clones, and then expanding and freezing the selected clones took more than 3 weeks.

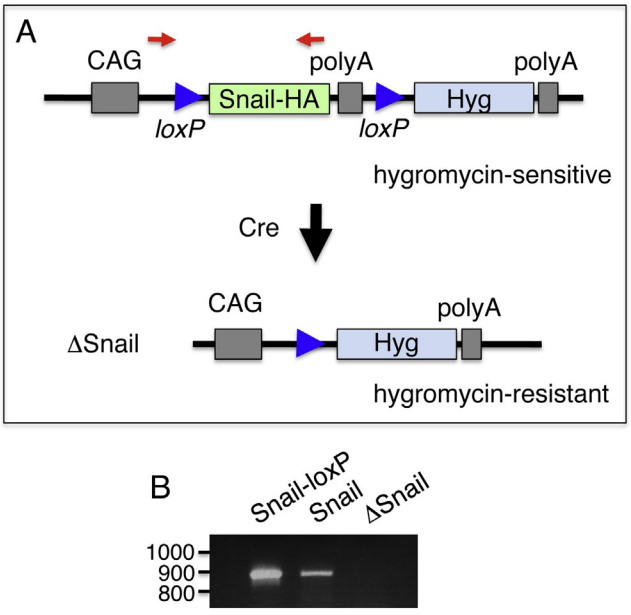

Fig. 1. (A) Schematic representation of the Snail-loxP vector and Cre-mediated removal of Snail-HA. The CAG promoter, comprising the CMV enhancer and the chicken β-actin promoter, drives expression of the downstream gene. The first gene, Snail-HA, followed by a rabbit β-globin polyadenylation signal (pA) is flanked by loxP sites (blue arrowheads) and is removed by Cre excision. The second gene, expressed only after Cre excision, is the hygromycin-resistance gene (Hyg), followed by the SV40 polyadenylation signal. Red arrows represent the primer set used for PCR identification of the Snail sequence. (B) Identification of the Snail sequence in the genomic DNA by PCR. Genomic DNA was isolated from Snail+ and ΔSnail cells and subjected to PCR using the primers indicated in panel (A). Snail-loxP vector DNA was used as a positive control.

2.3. Identification of the transgene

Genomic DNA was isolated by the method described [21], purified using a QIAquick DNA purification kit (Qiagen, Germantown, MD, USA), and used as a template for PCR reactions with GoTaq DNA polymerase (Promega, Madison, WI, USA). The primers for the PCR amplification of the 889 bp sequence, which includes a part of the vector as well as human Snail, were 5′-TGCTGTCTCATCATTTTGGCAA-3′ and 5′-GTCGTAGGGGTAGCCGATAT-3′.

2.4. Antibodies

A rat mAb against HA and a rabbit antibody targeting occludin were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and Zymed Laboratories (South San Francisco, CA, USA), respectively. A mouse mAb against vinculin and FITC-labeled phalloidin were purchased from Sigma (St. Louis, MO, USA). Other mAbs were purchased from Transduction Laboratories (Lexington, KY, USA). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

2.5. Immunofluorescence

Cells grown on coverslips were fixed, incubated with primary and secondary antibodies as previously described [17], and analyzed using an Olympus fluorescence microscope (Tokyo, Japan) equipped with a CD72 camera (Olympus).

2.6. Immunoblotting

Immunoblot analysis was carried out as previously described [17]. Briefly, proteins were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk, and then incubated with a primary antibody, followed by incubation with a peroxidase-conjugated secondary antibody. Bound antibody was visualized by enhanced chemiluminescence (ECL; Amersham International, Little Chalfont, UK).

2.7. Dissociation assay

Cells were washed with PBS and then incubated for 2 h in DMEM supplemented with 10% FCS containing 2.4 U/ml of dispase (Gibco). Detached cells were subjected to mechanical stress by pipetting with a 1 ml pipette as previously described [22].

2.8. Wound-healing assay

Cells were plated on 35 mm dishes and grown to confluency. Then, the cell monolayer was manually scratched with a pipette tip, washed with PBS, and incubated for 24 h. A phase contrast microscope was used to photograph the cells at 0 and 24 h after performing the scratch [22].

2.9. Gene expression microarray and data analysis

Total RNA was prepared from neo, Snail+, and ΔSnail MDCK cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and purified as previously described [22]. cRNA was amplified and labeled using a Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA) and hybridized to a 44K Agilent 60-mer oligomicroarray (Canine Oligo Microarray Kit). The hybridized microarray slides were scanned using an Agilent scanner. The relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (version 9.5.1.1). Microarray data analysis was supported by Cell Innovator (Fukuoka, Japan).

2.10. DNA methylation analysis

Genomic DNA (~0.75 μg) was treated with sodium bisulfite using the EpiTect system (Qiagen, Germantown, MD, USA). Bisulfite-converted DNA (~400 ng) was used as a template for PCR amplification of the CpG islands in the E-cadherin promoter. The primer pairs were sense (5′-GTTAAAATTAGTTAAAGAGTTAAAGT-3′) and antisense (5′-TACCTACAACAACAACAACAAC-3′). PCR products were purified from a 1% agarose gel using a Gel Extraction Kit (Qiagen) and cloned into the pGEM-T Easy vector (Promega). Four randomly selected clones from each sample were selected for sequencing.

3. Results

3.1. Snail-loxP vector

The vector contains the CAG promoter, a loxP-flanked region containing the HA-tagged Snail (Snail HA) gene followed by a β-globin polyadenylation signal (polyA), a hygromycin gene, and a neo gene under the control of the herpes simplex virus thymidine kinase (tk) promoter (Fig. 1A). The vector was designed so that Cre activity would result in the excision of the Snail HA gene, which would consequently permit the expression of the hygromycin-resistance gene (Fig. 1B). The CAG promoter allowed for strong expression of the Snail protein, and the neo gene provided resistance to G418.

3.2. Expression of Snail-loxP in MDCK cells induces EMT

MDCK cells are epithelial cells that are sensitive to the induction of EMT by either TGF-β [23] or transcription factors such as Snail [17] or ZEB2 [24]. We introduced the Snail-loxP vector into MDCK cells to generate stable transfectants after selection in G418. The ectopic expression of Snail (Fig. 2A) induced morphological changes whereby the cells became fibroblastic (Fig. 2A). Snail was detected in the nucleus as revealed by immunodetection of the HA-tag appended to its C-terminus (Fig. 2B). Immunoblot analysis revealed the downregulation of E-cadherin and occludin, and the upregulation of N-cadherin and fibronectin (Fig. 3A). These changes are characteristic of cells undergoing EMT [1,2]. Consistent with the immunoblot analysis, membrane staining of E-cadherin and occludin was lost in Snail+ cells (Fig. 3B). Rather than the cortical actin membrane staining observed in the control (neo) cells, the Snail+ cells had stress fibers in the cytoplasm, as revealed by phalloidin staining (Fig. 3B).

Cells undergoing EMT lose cell–cell adhesion and increase their motility. Cell dissociation assays demonstrated significant differences in the cell–cell adhesion of MDCK cells transfected with the control (neo) vector and MDCK cells transfected with the Snail-loxP vector (Fig. 3C). Similarly, wound-healing assays demonstrated significant differences in the directional migration and motility of MDCK cells transfected with either the control or the Snail-loxP vector. MDCK cells expressing Snail-loxP closed the scratched area faster than did cells expressing the control (neo) gene (Fig. 3D). Furthermore, a Matrigel invasion assay revealed that the MDCK

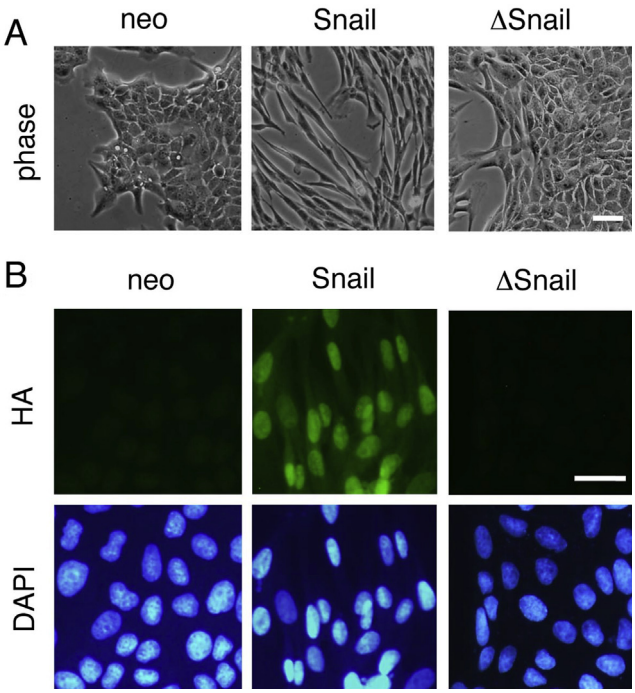

Fig. 2. Expression of Snail-loxP induces EMT and Cre-mediated excision of the Snail sequence results in MET in MDCK cells. (A) Phase contrast microscopy shows that the expression of Snail-loxP induced morphological (epithelial to fibroblastic) changes. Cre-mediated excision of the Snail sequence reverted the morphology. After Cre-mediated excision of the Snail sequence, ΔSnail cells became epithelial cell-like. (B) Staining with an anti-HA antibody and DAPI revealed the nuclear localization of Snail-HA. Bars, 25 μm.

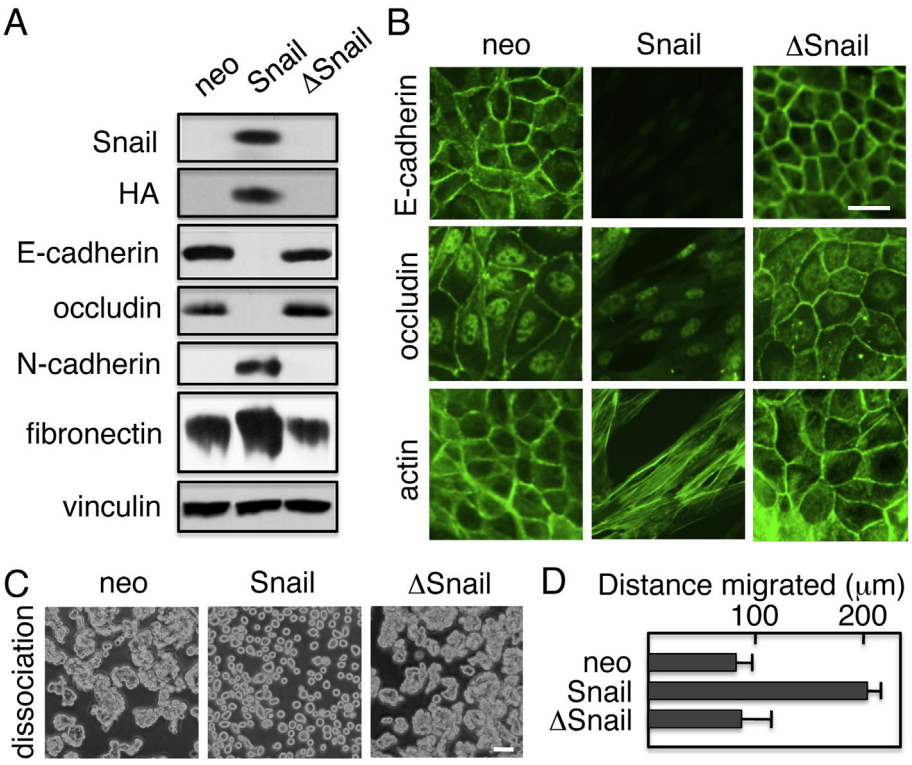

Fig. 3. Reversibility of Snail-induced molecular changes upon Cre-mediated deletion of Snail. (A) Immunoblot analysis revealed that the expression of Snail-loxP resulted in the downregulation of E-cadherin and occludin and the up-regulation of N-cadherin and fibronectin. Vinculin was used as a loading control. (B) Immunostaining revealed that Snail expression downregulates E-cadherin and occludin expression. The occludin antibody reacts with nuclear material(s) that is not occludin. The cortical actin pattern disappears and stress fibers are formed in Snail+ cells. (C) Snail+ cells exhibit increased dissociation, but ΔSnail cells show decreased dissociation. (D) A wound-healing assay indicated that Snail+ cells increased their migratory activity approximately two-fold as compared to control cells. Bars, 25 μm.

cells expressing Snail had a greatly increased invasive capacity (data not shown). Together, these results supported the hypothesis that Snail-loxP drives EMT in MDCK cells.

3.3. *Cre-mediated excision of Snail-loxP results in EMT reversal (MET)*

When Cre was transiently expressed in Snail+ cells and hygromycin-resistant cells were isolated, a variety of analyses revealed that Snail expression was lost at both the mRNA and protein levels. Specifically, PCR analysis of genomic DNA revealed that an 889 bp sequence, which includes a part of the vector and human Snail, was detected in Snail+ and lost in ΔSnail cells (Fig. 1B). Furthermore, the nuclear staining of Snail protein as detected by an anti-HA antibody in Snail+ cells was no longer observed in ΔSnail cells (Fig. 2B) and immunoblot analysis revealed that Snail protein expression was lost in ΔSnail cells (Fig. 3A). Moreover, the fibroblastic morphology observed in the Snail+ cells was reverted to an epithelial morphology in ΔSnail cells (Fig. 2A).

Immunoblot analysis revealed that concomitant with loss of Snail expression, E-cadherin and occludin protein expression was recovered and N-cadherin and fibronectin protein expression was lost (Fig. 3A). In ΔSnail cells, the recovered E-cadherin and occludin proteins were localized to the plasma membrane, actin stress fibers were lost, and cortical membrane staining of actin was restored (Fig. 3B). Dissociation assays showed that ΔSnail cells exhibited much stronger cell adhesion than Snail+ cells, consistent with a return to epithelial morphology with established cell–cell contacts (Fig. 3C). Wound-healing assays demonstrated that ΔSnail cells had a decreased migratory ability as compared to Snail+ cells (Fig. 3D). These results indicated that after Cre-mediated deletion of the loxP-flanked Snail gene, mesenchymal characteristics were lost and epithelial traits were gained.

Previous studies reported the upregulation of transcription factors, e.g., Slug and ZEB1, upon Snail expression [25]. Using an Agilent Whole Canine Genome microarray, we compared the gene expression profiles among control (neo), Snail+, and ΔSnail MDCK cells. The mRNA expression levels of Slug, ZEB1, and ZEB2 were significantly increased in MDCK cells expressing Snail and were returned to their original levels in ΔSnail cells (Table 1). Thus, it is very likely that the expression of Snail in MDCK cells induced the upregulation of Slug, ZEB1, and ZEB2, and that these transcription factors cooperated to downregulate E-cadherin expression and

Table 1
Changes in relative expression levels of epithelial and mesenchymal markers and EMT-related transcription factors upon Snail expression and Cre-mediated elimination of Snail (ΔSnail) in MDCK cells.

Gene	Genbank accession	neo → Snail+	Snail+ → ΔSnail
E-cadherin	AF330162	−2.81	2.24
Occludin	NM_001003195	−2.93	3.13
N-cadherin	XM_537293	2.69	−4.01
Fibronectin	U52105	5.16	−5.06
Vimentin	XM_851385	5.07	−5.75
Slug	NM_001097981	4.63	−4.44
ZEB1	XM_854899	4.36	−4.24
ZEB2	XM_541029	3.09	−3.22

The gene expression profiles of control (neo), Snail+, and ΔSnail MDCK cells were compared using an Agilent Whole Canine Genome microarray. Data are presented as the relative intensities of signals from Snail+ cells to those from control (neo) cells, and the relative intensities of signals from ΔSnail cells to those from Snail+ cells.

induce EMT. The removal of Snail-loxP gene by Cre expression downregulated the levels of these critical transcription factors.

3.4. E-cadherin promoter was not methylated in Snail MDBK cells

Previous analysis of the E-cadherin gene revealed that the proximal promoter of E-cadherin contains CpG islands, which are targets for methylation, and that TGF-β-induced EMT results in methylation of the E-cadherin promoter [10,11]. Therefore, we examined the methylation status of the E-cadherin promoter in ΔSnail MDCK cells. However, no significant de novo DNA methylation was detected at the E-cadherin promoter in Snail+ cells as compared with control (neo) cells as measured by bisulfite sequencing (Fig. 4). Thus, although Snail expressed in MDCK cells induced the downregulation of E-cadherin expression and EMT, Snail expression alone was not sufficient to induce methylation of the E-cadherin promoter. In other words, silencing of the E-cadherin gene by DNA methylation is not necessary to downregulate E-cadherin expression.

4. Discussion

In this study, we showed that the ectopic expression of the Snail-loxP gene in MDCK cells induced changes that are characteristic of EMT. The observed changes included the acquisition of a fibroblast-like morphology, the decreased expression of E-cadherin and occludin, and the increased expression of N-cadherin and fibronectin. Since these changes are hallmarks of EMT [1,2], MDCK cells expressing Snail-loxP could be classified as cells that had undergone EMT. As further evidence of EMT, Snail+ cells acquired the ability to migrate and dissociate. More importantly, all of these changes were reversed through the removal of the Snail-loxP gene by Cre expression. Thus, EMT induced by forced expression of Snail in MDCK cells was reversible.

In another study, Snail was expressed in MDCK cells using a tetracycline-inducible expression system [26]. However, Snail expression did not result in overt EMT, and the reversibility of Snail's effects was not thoroughly examined. Other investigators examined a similar tetracycline-inducible expression system in the colorectal cancer cell line, DLD-1, to show that the conditional expression of Snail induces an epithelial dedifferentiation program that coincides with a drastic change in cell morphology [27]. That study also did not examine the reversibility of Snail's effects.

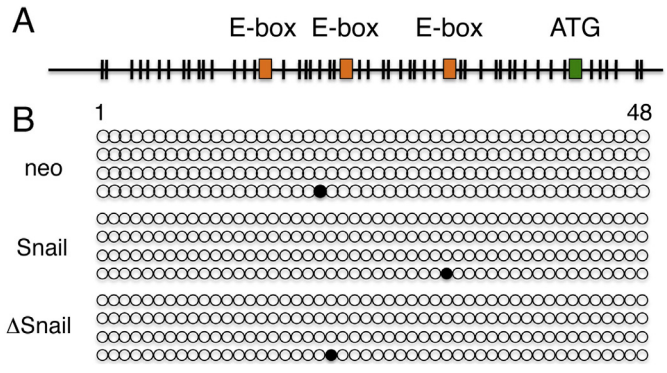

Fig. 4. Ectopic expression of Snail in MDCK cells does not induce DNA methylation of the E-cadherin promoter. (A) Diagram showing the position of three E-boxes (orange boxes; −187 to −182, −137 to −132, and −88 to −83) and 48 CpG dinucleotides analyzed within the E-cadherin promoter region (vertical bars). (B) Genomic DNA was isolated from control (neo), Snail+, and ΔSnail cells, and methylation of the E-cadherin promoter was analyzed by bisulfite sequencing. Methylated and unmethylated dinucleotides are indicated as filled and open circles, respectively.

The addition of tamoxifen to immortalized human mammary epithelial cells expressing a tamoxifen-activatable form of Snail induced EMT [28]. Although the reversibility of Snail's effects were not rigorously tested, the treated cells retained the ability to form ~10-fold more colonies in soft agar suspension culture as compared to the control, untreated cells. These effects persisted even after the withdrawal of tamoxifen, raising the possibility that the cells had undergone irreversible changes. In another experiment, a similar Snail-ER fusion protein was used as a tamoxifen-activatable form to identify Snail-regulated genes in oral keratinocytes [29]. This latter study also did not examine the reversibility of Snail's effects.

Stable silencing of Snail expression by shRNA efficiently derepresses E-cadherin expression and leads to MET in the stably transfected MDCK-Snail cell system. Stable interference of endogenous Snail in carcinoma cell lines leads to a dramatic reduction of in vivo tumor growth [14]. Although the abrogation of Snail expression mediated by the shRNAs seems to be highly specific and occurs without affecting the homologous zinc-finger factor, Slug, possible off-target effects of the shRNAs cannot be excluded, because rescue experiments with cDNA constructs that are not silenced by shRNAs were not performed. However, the results of the present study reinforce the conclusion of the shRNA study that Snail-induced EMT is reversible.

Downregulation of E-cadherin expression in human cancers through methylation of the E-cadherin promoter region has been reported [30]. Furthermore, promoter hypermethylation and increased Snail expression were found to be common (>35%), and to be strongly associated with reduced/negative E-cadherin expression [31]. Thus, epigenetic changes, including E-cadherin promoter hypermethylation and transcriptional repression, are the main mechanisms involved in E-cadherin downregulation in most carcinomas and carcinoma-derived cell lines [32]. It has been shown that methylation of the E-cadherin promoter regions occurs after prolonged treatment with TGF-β [10,11], which induces expression of Snail [8], Slug [9], and ZEB1 [13,33]. EMT induced by prolonged (12 days) treatment of TGF-β becomes irreversible [11]. In the present study, we showed that the promoter region of E-cadherin is not methylated in Snail-loxP stable transfectants, even after more than 3 weeks of culture. These cells did exhibit exogenously induced Snail expression, as well as the increased expression of other EMT-inducing transcription factors, e.g., Slug, ZEB1, and ZEB2. Therefore, the increased expression of these transcription factors was not sufficient to induce methylation of the E-cadherin promoter. Additional factor(s) that are induced by TGF-β treatment, but are not induced by Snail expression, seem to be necessary. Lack of methylation of the E-cadherin promoter region is consistent with the observation that EMT induced by Snail-loxP expression is reversible.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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Transparency document

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