

ORIGINAL ARTICLE

Suppression of SCARA5 by Snail1 is essential for EMT-associated cell migration of A549 cells

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Accumulating evidence indicates that epithelial-to-mesenchymal transition (EMT) might be a key event for cancer progression. The upregulation of Snail1, one of the most extensively studied EMT regulators, has been implicated in cancer metastasis, but the underlying mechanisms remain unclear. This study aims to identify that Snail1 targets regulating EMT-associated cancer cell migration. Human lung carcinoma A549 cells were treated with transforming growth factor beta 1 (TGF- β 1), and EMT-associated phenotypic and functional alterations were monitored. TGF- β 1 induced typical EMT-like morphological changes, 'cadherin switching' and cell migration in A549 cells. TGF- β 1 stimulation induced rapid and persistent upregulation of Snail1. Moreover, Snail1 upregulation was required for EMT-associated cell migration. Several metastasis suppressors with putative Snail1-binding sites in their promoters were dramatically repressed in A549 cells during TGF- β 1-induced EMT. Gain- and loss-of Snail1 function experiments demonstrated that scavenger receptor class A member 5 (SCARA5) was negatively regulated by Snail1. Importantly, SCARA5 downregulation was essential for EMT-induced migration in A549 cells. The chromatin immunoprecipitation assay revealed that Snail1 could bind to the E-box elements in SCARA5 promoter, implying that SCARA5 is a direct Snail1 target modulating cancer cell mobility during EMT. In addition, we showed that DNA methyltransferase 1 was physically associated with Snail1 to silence SCARA5 expression with an unidentified DNA methylation-independent mechanism, suggesting the complexity of Snail1-mediated epigenetic regulation. Collectively, our data demonstrated that EMT-regulator Snail1 suppresses the expression of SCARA5 to promote cancer progression, highlighting the possibility to target Snail1 and SCARA5 for cancer treatment.

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INTRODUCTION

Metastasis is the major cause of cancer-associated death.¹ Metastasis occurs as a multistep process during which cancer cells detach from the primary tumor and intravasate circulation to disseminate and to invade surrounding tissues to form the secondary tumors.^{2–5} Emerging evidence supports that epithelial-to-mesenchymal transition (EMT) occurs at the invasive front of many epithelial cancers and is a crucial event for cancer metastasis.^{6–8} Similar to the EMT during embryonic development, EMT in cancer cells also reduces intercellular adhesion and increases cell motility, endowing epithelial cancer cells with migration and invasive properties.^{9,10} EMT-like subpopulation in the carcinoma moves much more rapidly and intravasates into the blood vessels more frequently *in vivo* than the collective clusters.¹¹ Understanding the molecular mechanisms modulating EMT in epithelial cancer cells should be important for the identification of novel targets to control cancer progression.

The transcriptional repressor Snail1 is a master EMT regulator induced by various EMT inducers.^{12,13} Upregulation of Snail1 in carcinoma cells decreases the expression of epithelial markers, induces apoptotic resistance and enhances cell mobility, which may increase the malignancy of cancer cells.¹⁴ Elevated Snail1 expression is associated with disease progression in various types of cancers, including pancreatic, breast, ovarian and liver cancers.^{8,15–17} It is well established that Snail1 can directly silence

the transcription of some epithelial markers, leading to the loss of the epithelial phenotype in cancer cells. A number of pro-apoptotic genes have also been identified as Snail1 targets.^{18–21} Thus, downregulation of these genes may contribute to the apoptotic resistance in cancer cells undergoing Snail1-mediated EMT. Although numerous studies show that Snail1 can enhance cancer cell migration and invasion, molecules targeted by Snail1 to modulate cancer cell mobility remain largely unclear.

The important role of Snail1 in EMT regulation has attracted much attention to understand the mechanisms by which Snail1 mediates transcriptional repression. It has been speculated that epigenetic modifications, the reversible modifications in DNA or histones specifying gene expression, can dynamically regulate cancer EMT during disease progression.²² The DNA-binding c-terminus of Snail1 has four zinc-fingers, which recognize the E-box consensus sequences in the promoters of its target genes, whereas the regulatory N-terminus recruits other proteins to define snail1 target expression. An increasing number of chromatin-modifying proteins has been found to interact with Snail1, creating a transcriptionally repressive environment on the promoters of Snail1-regulated genes.^{23–27} For example, lysine-specific demethylase 1 (LSD1) and histone deacetylases bind to the Snail/Gfi domain of Snail1 for histone H3 at lysine 4 (H3K4) demethylation and histone deacetylation, respectively,^{25,27} removing transcriptionally active chromatin to

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initiate gene silencing. On the other hand, the polycomb repressive complex 2 cooperates with Snail1 to assemble a transcriptionally repressive complex mediating the trimethylation of lysine 27 in histone H3 (H3K27).^{24,26} DNA methyltransferases (DNMTs) are the most recently identified Snail1-associated chromatin-modifying proteins, which may induce DNA methylation to mediate stable repression of epithelial markers in cells undergoing EMT induced by chronic transforming growth factor beta 1 (TGF- β 1) exposure.²³ The spatial-temporal-specific interactions between chromatin proteins with Snail1 seem to occur in a cellular context-dependent manner, suggesting that Snail1-mediated epigenetic modifications are highly regulated to coordinate the EMT program.

In the current study, using TGF- β 1-induced EMT in human lung cancer A549 cell line as a model, we show that Snail1 is an important mediator for TGF- β 1 signaling to induce EMT and to promote cancer cell migration. Snail1 represses the transcription of scavenger receptor class A member 5 (SCARA5), a recently identified tumor metastasis suppressor inhibiting focal adhesion kinase-1 (FAK) activation.²⁸ Interestingly, Snail1 seems to cooperate with DNA methyltransferase 1 (DNMT1) to silence SCARA5 expression in a DNA methylation-independent manner. Moreover, we provided evidence showing that the prevention of SCARA5 downregulation significantly attenuates the EMT-associated migration of A549 cells induced by TGF- β 1, indicating that SCARA5 is novel target silenced by Snail1 to enhance cancer cell mobility during EMT.

RESULTS

TGF- β 1 induces EMT in A549 cells

Human non-small cell lung cancer cell line A549 was cultured in the medium with or without TGF- β 1 (5 ng/ml) for 48 h. In the absence of TGF- β 1, A549 cells maintained typical epithelial morphology and were organized in compact islets during the culture. The majority of TGF- β 1-treated A549 cells underwent

EMT-like elongation to become fibroblast-like spindle-shaped cells (Figure 1a). The distribution of filamentous actin was visualized by rhodamine-phalloidin staining. In untreated A549 cells, filamentous actin filaments were tightly associated with cell-cell contact, showing a pericellular plasma membrane distribution. In contrast, filamentous actin was assembled into thick parallel bundles throughout the TGF- β 1-treated A549 cells (Figure 1a).

The loss of epithelial marker E-cadherin (E-cad) is a hallmark of EMT.¹² TGF- β 1 treatment induced progressive downregulation of E-cad mRNA in A549 cells (Figure 1b). At the protein level, E-cad expression only slightly decreased in A549 cells treated with TGF- β 1 for 24 h, but this downregulation was evident 48 h after TGF- β 1 exposure (Figure 1c). In contrast, TGF- β 1 treatment significantly induced the expression of mesenchymal marker N-cadherin at both mRNA and protein levels (Figures 1b, c). Thus, TGF- β 1 treatment is sufficient to induce EMT-associated 'cadherin switch' in A549 cells.

Increased cancer cell mobility is one of the best documented EMT-associated functional changes in cancer cells.^{29,30} Transwell assay showed that TGF- β 1 exposure significantly increased the number of migratory A549 cells, suggesting that TGF- β 1 treatment efficiently enhanced the mobility of A549 cells (Figure 1d). Flow cytometric DNA content analysis showed that the cell cycle of A549 cells remained unaffected during TGF- β 1 exposure. Consistently, the mRNA expression of multiple cell-cycle-related genes showed no significant expression changes in A549 cells after TGF- β 1 treatment for 48 h (Supplementary Figures S1A, B), demonstrating that TGF- β 1 induced A549 migration without promoting cell proliferation.

Snail1 is induced by TGF- β 1 to enhance A549 mobility during EMT. Upregulation of Snail1 has been reported in various EMT models.¹³ The quantitative reverse-transcription PCR assay revealed that TGF- β 1 stimulation rapidly increased Snail1 mRNA levels in A549

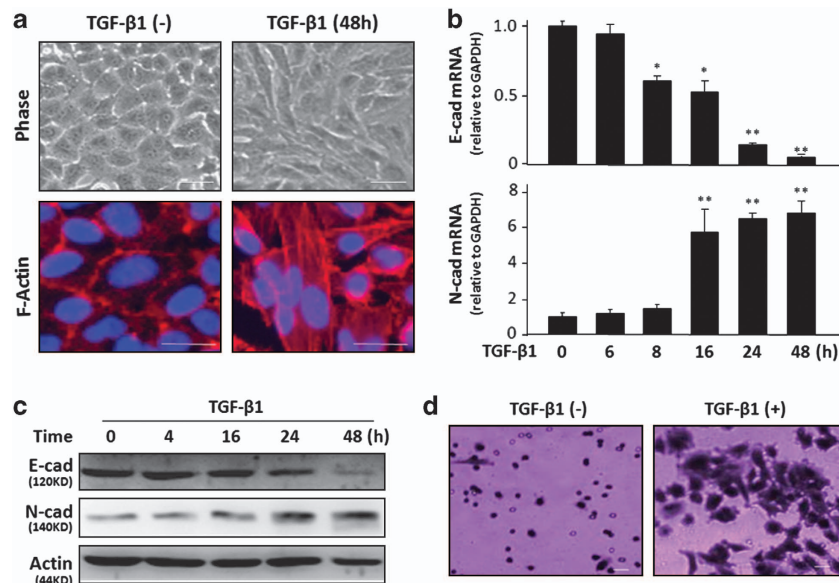


Figure 1. TGF- β 1 induces EMT-associated alterations in A549 cells. **(a)** The representative phase images of A549 cells cultured in the absence or presence of TGF- β 1 (5 ng/ml). TGF- β 1 exposure for 48 h induced EMT-like elongation in A549 cells. TGF- β 1 treatment induced the redistribution of filamentous actin (F-actin) in A549 cells, as determined by F-actin staining with Rhodamine-phalloidin. F-actin was stained in red and 4',6-diamidino-2-phenylindole stained nuclei in blue. Bars = 20 μ m. **(b)** Alterations of E-cad and N-cadherin (N-cad) mRNA expression in A549 cells after exposure to TGF- β 1 for various periods of time as assessed by quantitative reverse-transcription PCR (qRT-PCR). TGF- β 1 treatment induced progressive downregulation of E-cad and upregulation of N-cad. The qRT-PCR results represent an average of three independent samples. Values are means \pm s.e. ** P < 0.01 and * P < 0.05 compared with non-TGF- β 1-treated cells. **(c)** Cellular levels of E-cad and N-cad proteins in TGF- β 1-treated A549 cells as determined by western blot. Actin was blotted to ensure equal loading. Consistent with qRT-PCR analysis, TGF- β 1 also induced the downregulation of E-cad protein and upregulation of N-cad proteins, respectively. **(d)** The representative phase images of migratory A549 stained by crystal violet. TGF- β 1 treatment significantly increased the number of A549 cells that were capable of migrating through the membrane of the inserts. Bars = 30 μ m.

cells (Figure 2a). Interestingly, Snail1 upregulation in A549 cells could last for at least 48 h without the addition of new TGF- β 1, raising the possibility that TGF- β 1 stimulation might induce some epigenetic modifications to maintain Snail1 transcriptional activation in the cells. Western blot and immunofluorescent assays showed that the untreated A549 cells expressed Snail1 protein at a relatively low level. TGF- β 1 treatment for 4 h evidently induced Snail1 protein upregulation and this could persist for the following 48 h (Figure 2a and Supplementary Figure S2). The transcription of other EMT-associated transcriptional factors including Twist1/2, Zeb1/2 was not significantly changed by TGF- β 1 at time points tested in this study. However, Snail2 was also significantly induced by TGF- β 1 (Supplementary Figure S3), implying that Snail1 might not mediate all EMT-associated changes in TGF- β 1-treated A549 cells.

To explore the contribution of Snail1 upregulation to TGF- β 1-induced cell migration, A549 cells were transfected with a pool of specific small interfering RNAs (siRNAs) against Snail1. As shown in Figure 2b, Snail1 siRNAs reduced the basal level of endogenous

Snail1 in A549 cells. Moreover, Snail1 siRNAs significantly attenuated TGF- β 1-induced Snail1 upregulation. The expression of Snail2 mRNA expression was not affected by Snail1 siRNAs (data not shown), suggesting that the knockdown was specific to Snail1. As shown in Figure 2c, TGF- β 1-induced cell migration was significantly attenuated by Snail1 knockdown, indicating that the upregulation of Snail1 is important to enhance the migration of A549 cells during TGF- β 1-induced EMT.

TGF- β 1 treatment represses the transcription of multiple metastasis suppressors

Snail1 is a transcriptional repressor recognizing conserved E-box sequences, which might directly silence the expression of metastasis suppressors to enhance cell migration during EMT. We found that 6574 genes have four or more E-box elements in the 3 kb region around the transcriptional start sites.²¹ We reasoned that the transcription of Snail1 targets should be repressed in TGF- β 1-treated A549 cells in which Snail1 is

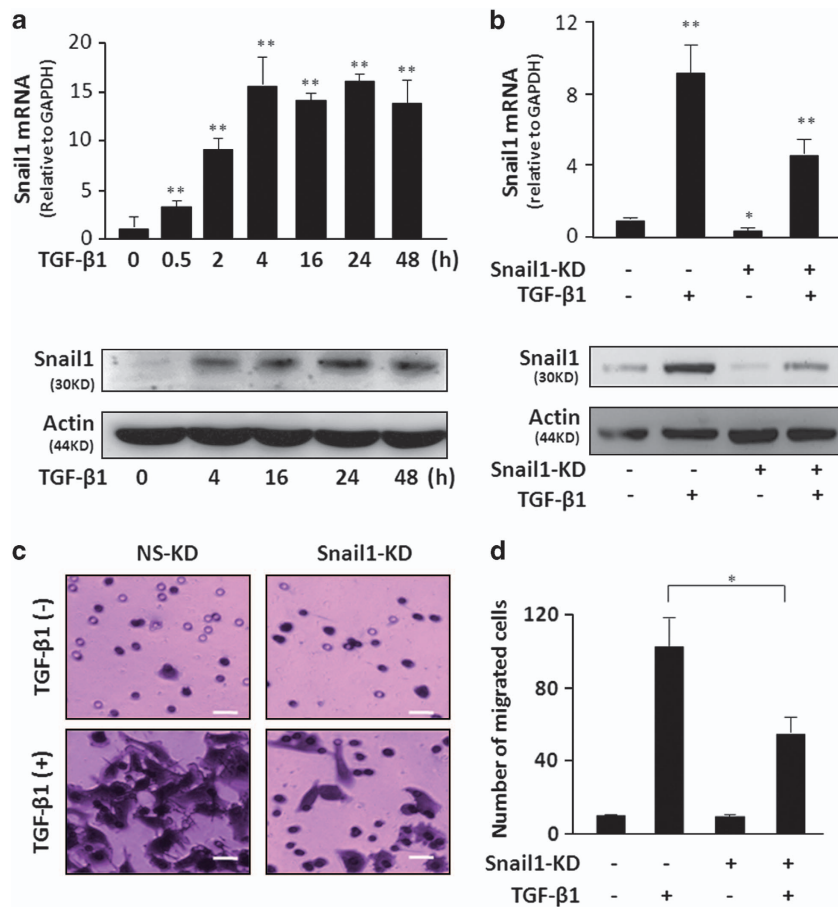


Figure 2. Snail1 mediates TGF- β 1 signaling to induce the migration of A549 cells. **(a)** Alterations of Snail1 mRNA levels in A549 cells after exposure to TGF- β 1 for indicated periods of time as assessed by qRT-PCR. TGF- β 1 treatment induced a rapid and persistent upregulation of Snail1. The qRT-PCR results represent an average of three independent samples. Values are means \pm s.e. $**P < 0.01$ compared with non-TGF- β 1-treated cells. Cellular Snail1 protein levels in TGF- β 1-treated A549 cells as determined by western blot. Actin was blotted to ensure equal loading. TGF- β 1 induced significant upregulation of Snail1 protein. **(b)** Snail1 knockdown was achieved by transient siRNA transfection. The knockdown efficiency was evaluated by both qRT-PCR and western blot. The Snail1 siRNAs not only decreased the endogenous expression of Snail1 but also attenuated the inductive effect of TGF- β 1 on Snail1 expression in A549 cells. The qRT-PCR results represent an average of three independent samples. Values are means \pm s.e. $**P < 0.01$ and $*P < 0.05$ compared with non-TGF- β 1 treated cells and/or cells transfected with control siRNA; Snail1-KD = snail1 siRNA knockdown. **(c)** and **(d)** Cells transfected with Snail1 siRNA or control siRNA were exposed to TGF- β 1 (5 ng/ml) for 48 h before transwell assay. The representative phase images of migratory A549 cells with crystal violet staining and the quantization of migratory A549 cells after siRNA transfection. TGF- β 1 treatment increased the number of migratory A549 cells transfected with control siRNA. This inductive effect was significantly attenuated in A549 cells transfected with Snail1 siRNA. Data represent an average of three replicates. Values are means \pm s.e. $*P < 0.05$ compared with non-TGF- β 1-treated cells and/or cells transfected with control siRNA. Snail1-KD = snail siRNA knockdown. Bars = 30 μ m.

induced. Using a microarray database for A549 cells treated with TGF- β 1 (<http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS3710>), we found that 683 genes were potentially downregulated by TGF- β 1. We examined the expression of 36 of these genes which have previously been identified as metastasis suppressors. The quantitative reverse-transcription PCR assay showed that TGF- β 1 treatment for 48 h significantly decreased the expression of nine genes in A549 cells (Figure 3a). We focused on SCARA5, melanoma inhibitory activity 2 (MIA2) and SAM pointed domain-containing Ets transcription factor (SPDEF), which showed the most dramatic downregulation (> five folds) to determine their expression alterations at different time points after TGF- β 1 exposure. All three genes showed a very similar downregulation pattern in response to TGF- β 1 stimulation, as shown in Figure 3b.

SCARA5 is silenced by Snail1 to induce cell migration during TGF- β 1-induced EMT

We examined whether Snail1 negatively regulates the transcription of MIA2, SCARA5 and SPDEF. As shown in Figure 4a, enforced expression of full-length Snail1 in A549 cells resulted in a 50% decrease of SCARA5 but did not significantly alter the expression

of MIA2 and SPDEF. However, Snail1 knockdown induced a fivefold upregulation of SCARA5 in A549 cells. The same treatment only induced a modest upregulation of MIA2 and SPDEF (Figure 4a). No significant alterations in Snail2 mRNA levels were detected in cells after Snail1 siRNA treatment (Figure 4a), confirming the specificity of the siRNA response. Next, we examined whether Snail1 can bind to the promoter of SCARA5. There are eight conserved E-box sequences in the 3 kb region around the SCARA5 transcriptional start site (Figure 4b). We designed six pairs of primers for chromatin immunoprecipitation (ChIP) assay. Each pair of primers covers a small DNA region (~150 bp) containing one or two E-box elements neighboring the transcription start site of SCARA5. An additional pair of primers covering a 150 bp region on E2F promoter devoid of E-box was included as a background control. The two fragments covering the three most upstream E-box elements in the SCARA5 promoter were significantly enriched by Snail1 antibody compared with the control immunoglobulin G. The other four fragments were only weakly enriched by the Snail1 antibody at similar levels to the control immunoglobulin G (Figure 4c). These data indicate that Snail1 may selectively bind to certain E-box elements in SCARA5 promoter.

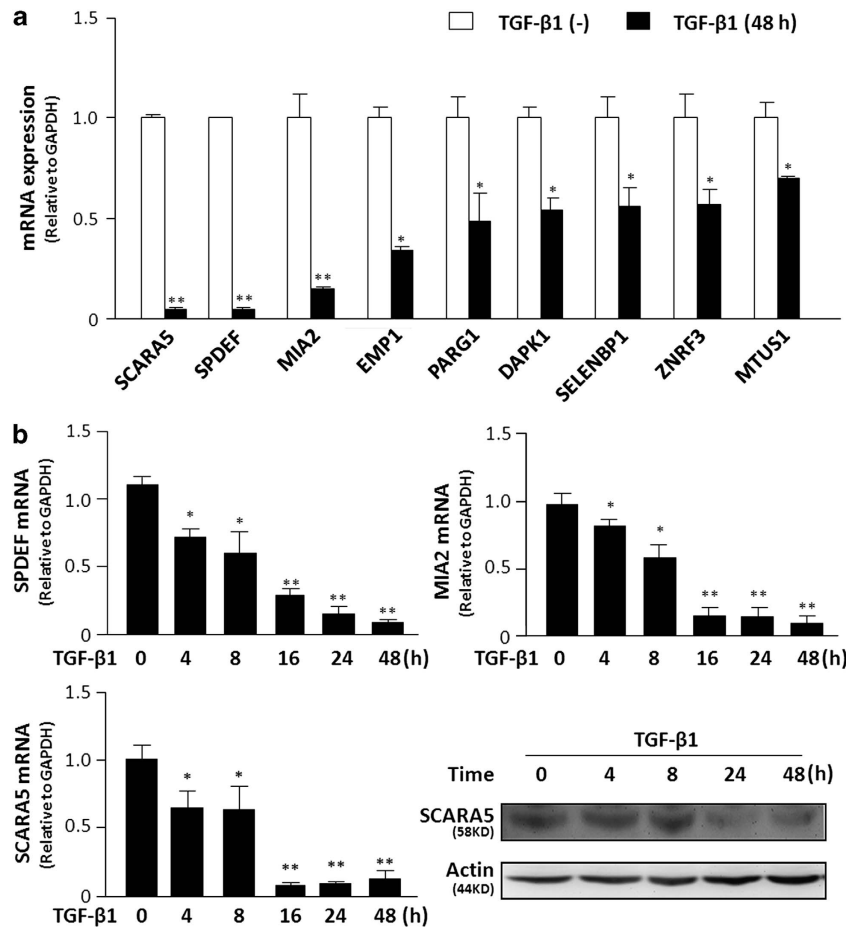


Figure 3. TGF- β 1 represses the transcription of multiple metastasis suppressors in A549 cells. **(a)** A549 cells were treated with TGF- β 1 for 48 h. The mRNA expression levels of multiple metastasis suppressors were determined by qRT-PCR. TGF- β 1 treatment induced statistically significant downregulation of nine genes. The qRT-PCR results represent an average of three independent samples. Values are means \pm s.e. $**P < 0.01$ and $*P < 0.05$ compared with non-TGF- β 1-treated cells. **(b)** Alterations of SCARA5, SPDEF and MIA2 mRNA expression in A549 cells after exposure to TGF- β 1 for various periods of time as assessed by qRT-PCR. TGF- β 1 treatment induced progressive downregulation of these genes in A549 cells. The qRT-PCR results shown represent an average of three independent samples. Values are means \pm s.e. $**P < 0.01$ and $*P < 0.05$ compared with non-TGF- β 1-treated cells. Cellular levels of SCARA5 protein in TGF- β 1-treated A549 cells were determined by western blot. Actin was blotted to ensure equal loading. The downregulation of SCARA5 in A549 cells was evident 24 h after TGF- β 1 stimulation.

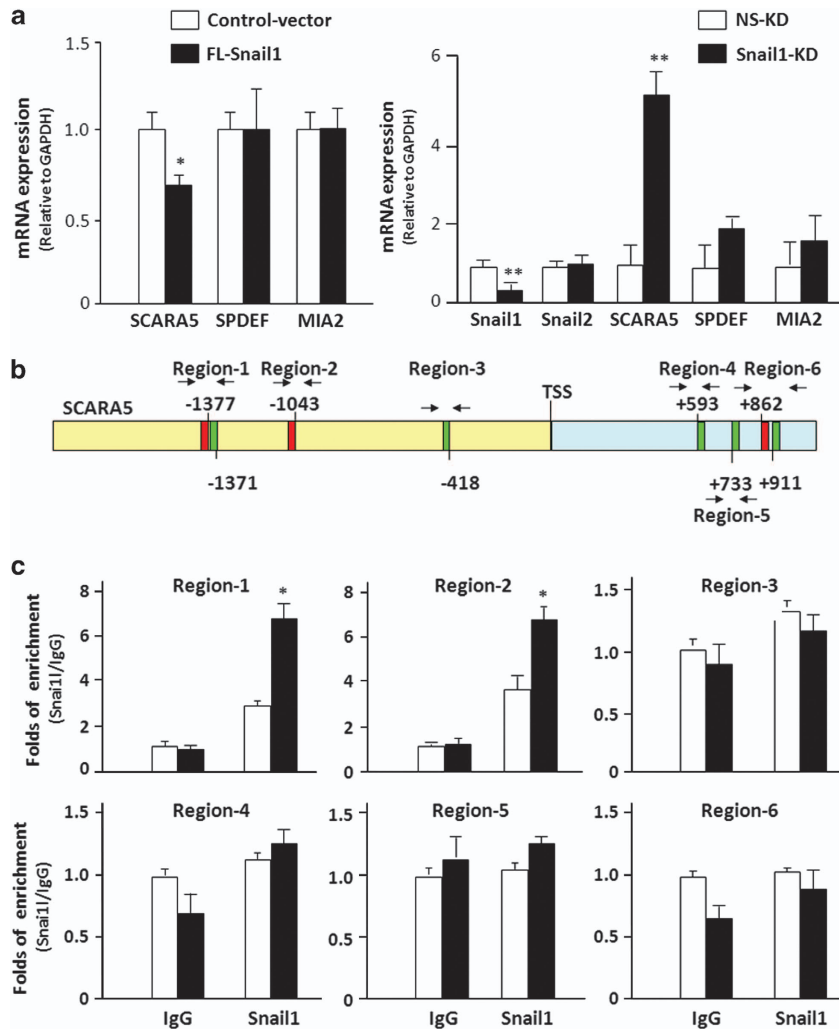


Figure 4. Snail1 directly represses SCARA5 transcription in A549 cells. (a) Alterations of SCARA5, SPDEF and MIA2 mRNA expression in A549 cells transfected with full-length Snail1-expressing plasmid or Snail1 siRNAs were determined by qRT-PCR. Enforced Snail1 expression decreased SCARA5 expression in A549 cells, but did not significantly affect the expression of SPDEF and MIA2. Snail1 knockdown in A549 cells resulted in dramatic upregulation of SCARA5. SPDEF and MIA2 were also modestly induced by Snail1 knockdown. The qRT-PCR results represent an average of three independent samples. Values are means \pm s.e. ** $P < 0.01$ and * $P < 0.05$ compared with the indicated control cells. NS-KD = non specific siRNA control, Snail1-KD = snail1 siRNA knockdown, FL-Snail1 = full-length Snail1. (b) The schematic of SCARA5 promoter. There are eight potential Snail1-binding E-box elements in 3 kb region around SCARA5 transcriptional start site. A total of six pairs of primers covering these sites were designed for ChIP assay. (c) The enrichment of six DNA fragments carrying different E-box elements in SCARA5 promoter by Snail1 immunoprecipitation. Only the two most upstream fragments were significantly pulled down by Snail1 antibody, as compared with control immunoglobulin G (IgG) antibody. Data represent an average of three replicates. Values are means \pm s.e. * $P < 0.05$ compared with the indicated control cells.

SCARA5 was initially identified as an iron delivery protein expressed by epithelial cells.³¹ Recent studies suggest that the loss of SCARA5 expression during cancer progression results in the aberrant FAK signaling pathway activation, enhancing cancer cell mobility and promoting tumor metastasis.²⁸ Knockdown of SCARA5 by a siRNA in A549 cell only slightly increased cell migration (Figures 5a, b). However, TGF- β 1-induced A549 migration was significantly decreased by enforced SCARA5 expression (Figures 5c, d), implicating that SCARA5 downregulation is essential but not sufficient to induce EMT-associated cell migration. The message and protein levels of SCARA5 in the cells with and without enforced SCARA5 expression were measured and shown in Supplementary Figure S4.

Non-coding RNA-a7 (ncRNA-a7) negatively regulates SCARA5 expression via enhancing Snail1 expression

The ncRNA-a7 is a newly identified regulatory ncRNA that positively regulates Snail1 transcription.³² We reasoned that

ncRNA-a7 might negatively control SCARA5 transcription in A549 cells. Interestingly, TGF- β 1 treatment induced a persistent upregulation of ncRNA-a7 in A549 cells (Figure 6a), suggesting its potential involvement in maintaining the EMT phenotype of TGF- β 1-treated A549 cells. Consistent with the previous study,³² ncRNA-a7 knockdown reduced endogenous Snail1 expression and attenuated TGF- β 1-induced Snail1 upregulation in A549 cells (Figure 6b). Conversely, ncRNA-a7 knockdown induced an upregulation of SCARA5 expression and attenuated the inhibitory effect of TGF- β 1 on SCARA5 transcription (Figure 6b), further supporting that SCARA5 is negatively regulated by Snail1 in A549 cells.

DNMT1 interacts with Snail1 to silence SCARA5 expression in a DNA-methylation-independent manner during EMT

The current model proposes that Snail1 cooperates with various chromatin-modifying proteins to silence the expression of target

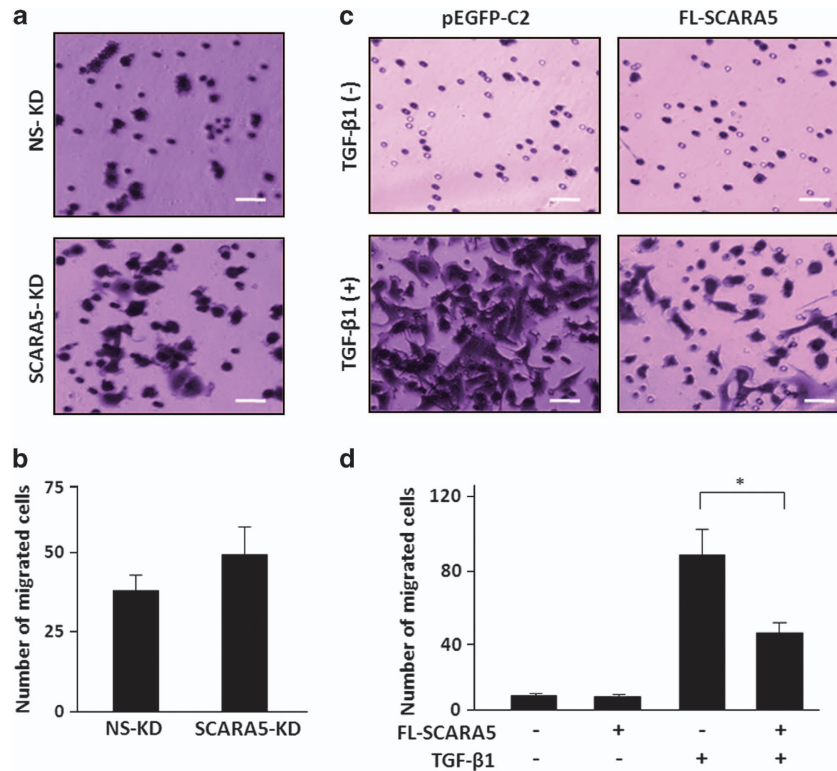


Figure 5. Downregulation of SCARA5 by TGF- β 1 is required to induce EMT-associated migration in A549 cells. The migration of A549 cells transfected with full-length SCARA5 or SCARA5 siRNA were determined by transwell assay. (a) and (b) SCARA5 knockdown alone in A549 cells did not significantly induce cell migration. (c) and (d) Enforced expression of full-length SCARA5 significantly inhibited TGF- β 1-induced migration of A549 cells. A549 cells were transfected with the full-length SCARA5 for 24 h and exposed to TGF- β 1 for additional 48 h, followed by Transwell Assay. Data represent an average of three replicates. Values are means \pm s.e. * P < 0.05 compared with indicated control cells. NS-KD = non specific siRNA control, SCARA5-KD = SCARA5 siRNA knockdown. FL-SCARA5 = full-length SCARA5. Bars = 30 μ m.

genes.³³ To identify the potential involvement of chromatin-modifying proteins in Snail1-mediated SCARA5 repression, A549 cells were treated for 48 h with inhibitors for polycomb repressive complex 2 (DZNep), LSD1 (Pargyline), histone deacetylases (Trichostatin A) and DNMT1 (azacitidine, AZA), followed by TGF- β 1 stimulation for 24 h. As shown in Figure 7a, only AZA pretreatment significantly increased SCARA5 expression in A549 cells and attenuated TGF- β 1-induced SCARA5 repression. Importantly, AZA pretreatment did not affect TGF- β 1-induced Snail1 upregulation (Supplementary Figure S5), indicating that the TGF- β 1 signaling pathway activation was not impaired by AZA pretreatment. These data suggest that DNMT1 might be the key chromatin-modifying protein recruited by Snail1 to silence SCARA5. Co-immunoprecipitation assay showed that the Snail1 antibody could simultaneously pull down Snail1 and DNMT1 proteins in TGF- β 1-treated A549 cells (Figure 7b), supporting the physical interaction between Snail1 and DNMT1 during EMT. Collectively, we concluded that Snail1 and DNMT1 might be assembled into a repressive complex to silence SCARA5 expression in A549 cells.

Promoter methylation is one of the major reasons responsible for SCARA5 repression in cancer patients with metastasis,²⁸ raising the possibility that Snail1 may recruit DNMT1 to methylate the SCARA5 promoter. We used the methylation-specific PCR assay to examine the methylation status of SCARA5 promoter in A549 cells. As shown in Figure 7c, SCARA5 promoter was partially methylated in A549 cells. However, TGF- β 1 treatment did not alter SCARA5 promoter methylation levels at all time points examined in this study, implying that Snail1 may cooperate with DNMT1 to silence SCARA5 expression with a DNA methylation-independent mechanism, at least during the early stage of EMT.

DNA methyltransferase 1-associated protein 1 (DMAP1) is a novel EMT repressor inhibiting Snail1 Expression

DMAP1 has an intrinsic repressive activity on transcription and can cooperate with DNMT1 to silence gene expression in a DNA-methylation-independent manner.³⁴ We knocked down DMAP1 with a siRNA in A549 cells to examine SCARA5 mRNA expression. Unexpectedly, DMAP1 knockdown induced a dramatic downregulation of SCARA5 in A549 cells (Figure 8a). Moreover, DMAP1 knockdown induced EMT-like morphological alteration in A549 cells (Figure 8b). These data promoted us to examine Snail1 expression in these cells. As shown in Figure 8c, Snail1 expression was significantly induced at both mRNA and protein levels in A549 cells with DMAP1 knockdown. Thus, rather than acting as a co-repressor for Snail1 to induce EMT-associated alterations, DMAP1 may be a novel Snail1 repressor to inhibit EMT initiation. Further study on the mechanism of DMAP1-mediated Snail1 repression may shed new insights on understanding the complicated mechanism of EMT regulation.

DISCUSSION

In the current study, we showed that the expression of SCARA5 was dramatically downregulated in a TGF- β 1-induced cancer cell EMT model. SCARA5 is one member of class A scavenger receptors that may be involved in iron delivery.³⁵ Unlike other scavenger receptors, the expression of SCARA5 is restricted to the epithelial population,³¹ suggesting that SCARA5 may have unique functions in epithelial cells. Thus, the remarkable downregulation of SCARA5 induced by TGF- β 1 may contribute to EMT-associated changes in cancer cells. Indeed, overexpression of SCARA5 efficiently

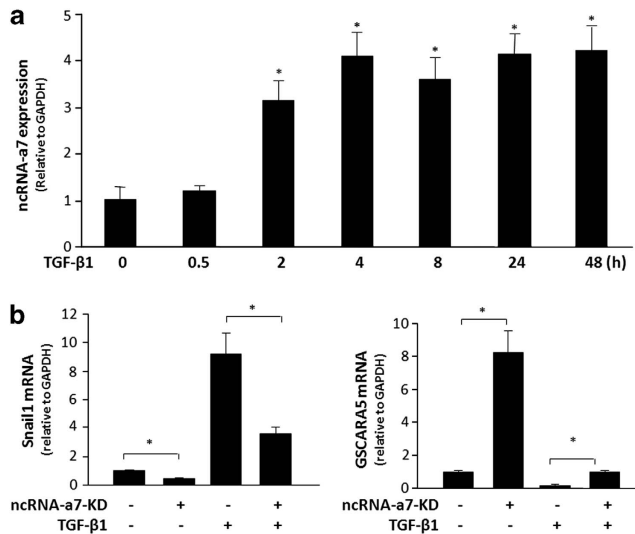


Figure 6. The regulatory effects of ncRNA-a7 on Snail1 and SCARA5 expression in A549 cells. **(a)** Alterations of ncRNA-a7 expression in A549 cells after exposure to TGF-β1 for various periods of time as assessed by qRT-PCR. TGF-β1 treatment induced a gradual upregulation of ncRNA-a7 in A549 cells. The qRT-PCR data represent an average of three independent samples. Values are means ± s.e. * $P < 0.05$ compared with non-TGF-β1-treated cells. **(b)** A549 cells transfected with control siRNA or ncRNA-a7 siRNA were treated with TGF-β1 for additional 24 h. Alterations of mRNA expression for Snail1 and SCARA5 were assessed by qRT-PCR. Knockdown of ncRNA-a7 decreased endogenous Snail1 expression and partially blocked TGF-β1-induced Snail1 upregulation. Knockdown of ncRNA-a7 significantly increased endogenous SCARA5 expression and attenuated TGF-β1-induced SCARA5 downregulation. The qRT-PCR results represent an average of three independent samples. Values are means ± s.e. * $P < 0.05$ compared with the indicated control cells. ncRNA-a7-KD = ncRNA-a7 siRNA knockdown.

attenuated TGF-β1-induced tumor cell migration in our EMT model, implying that downregulation of SCARA5 is a critical event to enhance the mobility of cancer cells undergoing TGF-β1-induced EMT.

A recent study found that the SCARA5 protein is physically associated with FAK at the cell membrane. This interaction inhibits FAK activation by preventing FAK phosphorylation on the Tyr-397 residue, the critical modification in mediating FAK autophosphorylation and intramolecular phosphorylation.²⁸ FAK has been known as a regulator for cell migration, and numerous clinical studies reported an aberrant FAK activation in various types of cancer patients with metastasis.^{36,37} Interestingly, FAK activation is also an important event to induce the migration of cells undergoing EMT.^{38–41} Thus, downregulation of SCARA5 by TGF-β1 may contribute to the induction of EMT-associated migration via modulating FAK activation in our model. However, it is important to point out that SCARA5 knockdown alone only enhanced A549 migration slightly, indicating that other genes regulated by TGF-β1 may be also important to increase cell mobility during EMT. Indeed, several other tumor metastasis suppressors, particularly MIA2 and SPDEF were also downregulated when A549 cells undergoing TGF-β1-induced EMT. Careful evaluation of the complex interplay between SCARA5 and these genes should provide additional information for understanding the mechanisms of how EMT promotes cancer metastasis.

Currently, little is known about the molecular mechanisms to regulate SCARA5 expression. In an attempt to identify novel Snail1 targets regulating cell migration during EMT, we found that there are multiple E-box sequences for potential Snail1 binding in the 3 kb region around the transcription start site of SCARA5. ChIP analysis revealed that Snail1 preferentially enriched DNA

fragments covering the three E-box elements located at –1377 nt and –1043 nt upstream of SCARA5 transcription start site, implying that SCARA5 is a direct target of Snail1. Consistently, we found that SCARA5 transcription was inversely correlated with Snail1 expression in A549 cells undergoing TGF-β1-induced EMT. Moreover, gain- and loss-of-function experiments confirmed the reverse relation between Snail1 and SCARA5 expression, further supporting that Snail1 acts as a transcriptional repressor to inhibit SCARA5 expression. Increasing evidence suggests that the interactions with chromatin-modifying proteins are essential for Snail1-mediated gene silencing. Similar to the results from a recent study,²³ we demonstrated the physical association between DNMT1 and Snail1. Consistently, the inhibition of DNMT1 by AZA significantly enhanced SCARA5 expression in A549 cells. In addition, it attenuated TGF-β1-induced downregulation of SCARA5, suggesting that DNMT1 might be the key chromatin-modifying protein cooperating with Snail1 to silence SCARA5 transcription. Snail1-associated DNMTs can methylate E-cad promoter in cells exposed to TGF-β1 for 9 days.²³ Our data showed that SCARA5 promoter was methylated in a subpopulation of untreated A549 cells, raising the possibility that constitutive expression of low level endogenous Snail1 may mediate the DNA methylation on SCARA5 promoter in these cells. However, TGF-β1 induced significant downregulation of SCARA5 in just a few hours after treatment, which is unlikely to be caused by DNA methylation. Indeed, we found that TGF-β1 treatment did not induce further DNA methylation on the SCARA5 promoter during the time course (48 h) examined in the current study, suggesting that the Snail1/DNMT1 complex can silence SCARA5 through an unknown DNA methylation-independent mechanism.

Several other chromatin-modifying proteins such as polycomb repressive complex 2, LSD1 and histone deacetylases have also been reported to cooperate with Snail1.^{24–27,42} However, we found that treatment with inhibitors for polycomb repressive complex 2, LSD1 and histone deacetylases did not affect SCARA5 expression in A549 cells regardless of TGF-β1 stimulation, suggesting that they are dispensable for Snail1 to repress SCARA5 transcription. These data should not be considered unexpected because that Snail1 may cooperate with chromatin-modifying proteins in a cellular context-dependent or target gene-specific manner. For example, Lin *et al.*²⁵ reported that Snail1 recruited LSD1 to silence E-cad expression in breast cancer cells. In contrast, McDonald *et al.*⁴³ showed that LSD1 was dispensable for E-cad downregulation but important to induce cell migration in TGF-β1-treated hepatocytes. It is possible that Snail1 is involved in the assembly of multiple distinct regulatory complexes in a spatial and temporal specific manner. Alternatively, Snail1 may only form a common large multiple-component complex for most target genes, but the gene-specific environment at the promoter region determines which particular proteins are essential to mediate Snail1 repressive function.

Another line of evidence to support that SCARA5 is a Snail1 target came from the loss-of-function experiment for ncRNA-a7 in A549 cells. ncRNA-a7 is a regulatory ncRNA located 135 kb downstream of Snail1. It has an enhancer-like function to facilitate Snail1 transcription.³² We found that TGF-β1 treatment induced a significant upregulation of ncRNA-a7 expression in A549 cells. The upregulation of ncRNA-a7 occurred a bit later than Snail1 upregulation after TGF-β1 stimulation, indicating that it is not necessary for the early induction of Snail1 by TGF-β1. However, ncRNA-a7 knockdown decreased the basal level of Snail1 expression and eventually attenuated TGF-β1-induced long-term Snail1 upregulation, implying that this molecule might be critical to determine the steady-state Snail1 transcriptional level. Importantly, we showed that ncRNA-a7 knockdown and Snail1 knockdown had very similar regulatory effects on SCARA5 expression and cell migration in A549 cells, further supporting that SCARA5 is a Snail1 target modulating cell mobility during EMT.

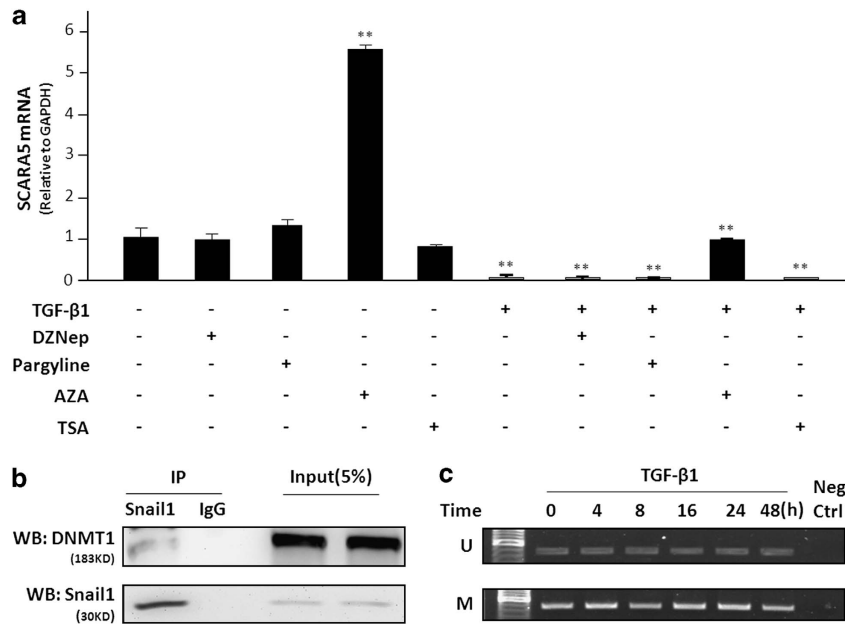


Figure 7. Snail1 cooperates with DNMT1 to silence SCARA5 expression in A549 cells. **(a)** The A549 cells were pretreated with indicated inhibitors for 48 h. Alterations of SCARA5 mRNA expression in pretreated A549 cells after exposure to TGF-β1 for 48 h were determined by qRT-PCR. Only AZA pretreatment significantly induced SCARA5 expression and attenuated TGF-β1-induced downregulation of SCARA5. The qRT-PCR results represent an average of three independent samples. Values are means \pm s.e. $**P < 0.01$ compared with the indicated control cells. **(b)** A549 cells were treated with TGF-β1 for 48 h to induce the expression of Snail1. Endogenous Snail1 was immunoprecipitated from TGF-β1-treated A549 cells. The bound Snail and DNMT1 were examined by western blotting. Ten percent input were loaded for blotted to ensure equal amount of proteins for immunoprecipitation. **(c)** A549 cells were treated with TGF-β1 for various periods of time as labeled. The SCARA5 promoter methylation status was determined by the methylation-specific PCR assay. SCARA5 promoter was methylated in subpopulation of A549 cells, but TGF-β1 treatment did not significantly change SCARA5 promoter methylation in A549 cells. U = unmethylated; M = methylated.

Under normal physiological conditions, EMT only happens in the early development stage, suggesting the existence of a precise expression control for EMT-associated transcriptional factors including Snail1.¹⁴ Most studies have focused on identifying transcriptional activators induced to enhance Snail1 expression during EMT.^{12,13} For example, in response to TGF-β1 stimulation, Smad2 and Smad3 (Smad2/3) proteins are activated by phosphorylation within 15–30 min and recruited to the target promoters to induce transcription.⁴⁴ Consistent with this view, TGF-β1 treatment induced a rapid upregulation of Snail1 transcription (<30 min) in A549 cells, which might be critical for EMT initiation. Yet, removing negative regulation might be an alternative mechanism to activate Snail1 expression in epithelial cells. We unexpectedly discovered a negative regulatory effect of DMAP1 on Snail1 transcription. DMAP1 has an intrinsic transcriptionally repressive activity and can associate with DNMT1 to silence transcription without inducing DNA methylation.³⁴ We originally speculated that DMAP1 might be recruited by the Snail1/DNMT1 complex to mediate DNA methylation-independent repression on SCARA5. In contrast to this hypothesis, DMAP1 knockdown induced a steady upregulation of Snail1 in A549 cells, accompanied by the EMT-like morphological alteration and dramatic downregulation of SCARA5. These data indicate that DMAP1 might be a novel Snail1 repressor. Interestingly, TGF-β1-induced Snail1 upregulation could last for at least 48 h in A549 cells, but TGF-β1 has a very short half-life time (1 ~ 2 min) and Smad2/3 phosphorylation level declines a few hours after TGF-β1 stimulation,⁴⁴ suggesting that other mechanisms should also be important to sustain Snail1 expression in A549 cells undergoing EMT. It is tempting to speculate that TGF-β1 treatment may relieve DMAP1-mediated repression of Snail1 to induce EMT in cancer cells. Nevertheless, our data showed that TGF-β1 treatment did not decrease DMAP1

expression in A549 cells. It would be of interest to further examine whether DMAP1 can directly interact with the Snail1 promoter and whether this interaction will be abolished by TGF-β1 treatment.

MATERIALS AND METHODS

Cell culture and EMT induction

A549 was purchased from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. To induce EMT, A549 cells were seeded at 70% confluence and cultured for 48 h in Dulbecco's modified Eagle's medium with 5% fetal bovine serum. TGF-β1 was added to a final concentration of 5 ng/ml.

Quantitative reverse-transcription PCR

Total RNA was extracted with TRIzol (Invitrogen, Grand Island, NY, USA). Reverse transcription was performed with SuperScript II Reverse Transcriptase Kit with random primers, following the manufacturer's standard protocol (Invitrogen). Quantitative reverse-transcription PCR was performed with ABI-Prism 7900HT (Applied Biosystems, Foster City, CA, USA) with the SYBR Green polymerase chain reaction master mix (Applied Biosystems). Primer sequence information is available upon request. Experiments were performed in triplicate and the values were normalized to GAPDH.

Western blot

Protein lysates were prepared in the presence of protease inhibitors and 20–40 μg of protein was separated on 10% SDS-polyacrylamide gel electrophoresis gels. Blots were probed with primary antibodies (diluted in 5% nonfat milk) against E-cad (Rat monoclonal, Sigma, St Louis, MO, USA), N-cadherin (Rabbit polyclonal, Cell Signaling, Danvers, MA, USA), Snail1 (Rabbit monoclonal, Cell Signaling), SCARA5 and β-actin (Rabbit polyclonal, Abcam, Cambridge, MA, USA).

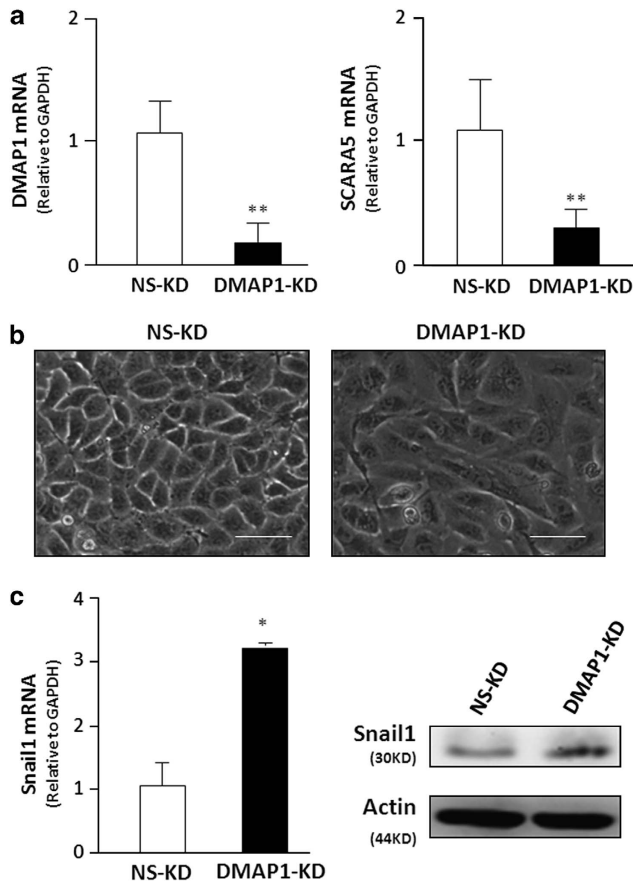


Figure 8. DMAP1 is a novel EMT repressor inhibiting Snail1 expression. (a) DMAP1 knockdown efficiency was evaluated at the mRNA level. The qRT-PCR assay showed that DMAP1 expression in A549 cells transfected with DMAP1 siRNA was 80% percent lower than that in cells transfected with a control siRNA. DMAP1 knockdown induced a significant upregulation of Snail1 mRNA in A549 cells. The qRT-PCR results shown represent an average of three independent samples. Values are means \pm s.e. $**P < 0.01$ compared with cells transfected with the non specific control siRNA. (b) Representative phase images of A549 cells 48 h after control siRNA or DMAP1 siRNA transfection. Control siRNA transfection did not affect the epithelial-like morphology of A549 cells. In contrast, DMAP1 knockdown induced EMT-like cell shape elongation in A549 cells. Bars = 30 μ m. (c) Alterations of Snail1 mRNA and protein expression in A549 cells transfected with DMAP1 siRNA or control siRNA were determined by western blot. Actin was blotted to ensure equal loading. DMAP1 knockdown in A549 cells induced significant upregulation of Snail1 in A549 cells. The qRT-PCR data represent an average of three independent samples. Values are means \pm s.e. $*P < 0.05$ compared with cells transfected with non specific control siRNA. NS-KD = non specific siRNA control; DMAP1-KD = DMAP1 siRNA knockdown.

Methylation-specific PCR assay

Genomic DNA was treated with EZ DNA Methylation-Gold Kit, following the manufacturer's standard protocol (Zymo Research, Irvine, CA, USA). To analyze the DNA methylation status of SCARA5 promoter, methylation-specific PCR was performed with bisulfate-treated genomic DNA, as previously reported.²⁸

Transwell assay

A total of 5×10^4 cells were suspended in 300 μ l fetal bovine serum-free Dulbecco's modified Eagle's medium and added to one insert with a permeable membrane at the bottom (8 μ m pore size). The inserts were placed into wells filled with 750 μ l Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. After incubation for 6 h, cells on the

lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Cells in four random microscopic fields ($\times 20$) were counted in triplicates.

Co-immunoprecipitation and ChIP

Co-immunoprecipitation assays were performed following the published protocol with small modifications.²⁶ A mouse monoclonal antibody against snail1 was used for immunoprecipitation and a rabbit polyclonal antibody against snail1 was used for blotting. ChIP assays were performed with a commercially available ChIP Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA) in accordance with the manufacturer's instructions. The extracts were sonicated on a Branson 450 Sonifier with a microtip at a power setting of 4 and a 60% duty cycle for 14 cycles (30 s sonication and 90 s rest). It sheared chromatin to a final average size of 500 bp. The chromatin fraction was immunoprecipitated overnight at 4 $^{\circ}$ C using a primary antibody against snail1 (mouse monoclonal, Cell Signaling). Fold enrichments relative to immunoglobulin G or the input control were quantified by real-time PCR using SYBR green PCR master mix (Applied Biosystems) and the ABI Prism 7000 system (Applied Biosystems).

Transient siRNA knockdown

Cells were seeded in 24-well plates at the density of 3×10^4 cells/well. Cells reached 40% confluence 16 h later. They were transfected with siRNAs at a final concentration of 100 nM using Lipofactamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. The cells were then cultured for 48 h to allow the knockdown. The control siRNA and Snail1 siRNAs (sc-38398) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SCARA5 siRNA: sense, 5'-GCUCCAUCUGAGGAUUCUU-3', antisense, 5'-GAAUCCUCACAGAUGGAGCUU-3';²⁸ DMAP1 siRNA: sense 5'-GUCUAUGCCUUGUCUACUUU-3', antisense 5'-AGUAGACAAGCAUAGACUU-3'; ncRNA-a7 siRNA: sense 5'-CCGAUUUGAGAGAGUGAGAUU-3', antisense, 5'-UCUCACUCUCUCAA AUCGGUU-3'.³²

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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