ALX1 Induces Snail Expression to Promote Epithelial-to-Mesenchymal Transition and Invasion of Ovarian Cancer Cells

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Abstract

Ovarian cancer is a highly invasive and metastatic disease with a poor prognosis if diagnosed at an advanced stage, which is often the case. Recent studies argue that ovarian cancer cells that have undergone epithelial-to-mesenchymal transition (EMT) acquire aggressive malignant properties, but the relevant molecular mechanisms in this setting are not well-understood. Here, we report findings from an siRNA screen that identified the homeobox transcription factor ALX1 as a novel regulator of EMT. RNA interference–mediated attenuation of ALX1 expression restored E-cadherin expression and cell–cell junction formation in ovarian cancer cells, suppressing cell invasion, anchorage-independent growth, and tumor formation. Conversely, enforced expression of ALX1 in ovarian cancer cells or nontumorigenic epithelial cells induced EMT. We found that ALX1 upregulated expression of the key EMT regulator Snail (SNAI1) and that it mediated EMT activation and cell invasion by ALX1. Our results define the ALX1/Snail axis as a novel EMT pathway that mediates cancer invasion. Cancer Res; 73(5); 1–10. ©2012 AACR

Introduction

Ovarian cancer is a highly metastatic disease and the most lethal of the gynecologic malignancies. Despite advances in cytotoxic therapies for numerous types of cancer, the current therapies available to patients with ovarian cancer in advanced stages have little effect, as evidenced by the poor 5-year survival rate (1, 2). To provide insight that will enable the development of new therapeutic strategies, it is crucial to elucidate the molecular mechanisms that promote the invasive and metastatic properties of ovarian cancer cells. Recent studies have shown that a morphologic conversion, known as epithelial-to-mesenchymal transition (EMT), is associated with the acquisition of malignant characteristics in ovarian cancer cells (3–8).

EMT is a complex multi-step process that occurs during embryonic development, tumor progression, and tissue fibrosis (9, 10). During EMT, epithelial cells lose many of their epithelial characteristics and adopt a mesenchymal appearance and mesenchymal characteristics, such as increased motility and invasiveness. Dynamic changes in gene expression and cytoskeletal re-organization are often observed during the process of EMT (11). One of the hallmarks of EMT is the suppression of E-cadherin, a transmembrane protein essential for cell–cell adhesions. The loss of E-cadherin expression not only disrupts cell–cell adhesion but also activates multiple pathways that induce cellular dissemination, invasion, and metastasis (12).

Extensive studies have revealed that EMT is governed by a variety of regulatory networks. EMT is triggered by extracellular stimuli, such as TGF-β, fibroblast growth factor, hepatocyte growth factor, and endothelin-1 (13, 14). The signaling pathways activated by these factors induce changes in cytoskeletal organization and disrupt cell–cell junctions. In addition to these signaling pathways, transcription factors, such as Snail, Slug, Twist, Zeb1, and SIP1, have been found to play crucial roles in promoting EMT (15). Among them, Snail is the most extensively studied and has been associated with tumor progression (16). Snail is a zinc-finger transcription factor that binds to E-box sequences located in the promoter regions of target genes (17, 18). Snail can also regulate transcription by modifying local chromosomal structures via the recruitment of histone deacetylase 1 (HDAC1) and HDAC2, as well as the co-repressor Sin3A (19). In addition to the well-known EMT inducers, recent studies have unveiled novel EMT-related transcription factors, such as HOXB7 (20), SIX1 (21, 22), FOXQ1 (23, 24), and FOXM1 (25). These findings indicate that EMT is regulated by a diverse set of transcription...
factors. Thus, the identification of additional factors will give further insight into the molecular mechanisms regulating EMT.

To search for novel regulators of EMT, we conducted a limited siRNA screen and found that Aristaless-like homeobox1 (ALX1), also known as Cart1 (26, 27), is important for the induction of morphologic changes in ovarian cancer cells. Previous studies have shown that ALX1 is required for neuronal or craniofacial development (28, 29), but the detailed physiologic role of ALX1 remains largely unknown. In this report, we show that ALX1 induces EMT and cell invasion in ovarian cancer cells by promoting Snail expression.

Materials and Methods

Materials and Methods have also been described in Supplementary Materials.

Cells, antibodies, and chemicals

All the ovarian cancer cells were cultured in RPMI supplemented with 10% FBS and antibiotics. K1, K2, NOS3, NOS4, TTOV, and TAOV cells were established in the Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine (Nagoya, Japan; ref. 30). ES-2 and RMG-II cells are originated from clear cell carcinoma and other cell lines are from serous carcinoma. 293T cells were maintained in Dulbecco’s Modified Eagle’s Media (DMEM) supplemented with 10% FBS with antibiotics. MCF10A cells were obtained from American Type Culture Collection and maintained in DMEM-F12 supplemented with 0.1 μg/mL cholera toxin (Sigma), 0.02 μg/mL EGF (PeproTech), 10 μg/mL insulin (Sigma), 0.5 μg/mL hydrocortisone (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin, and 5% horse serum (Invitrogen). Antibodies were obtained from the following companies: anti-E-cadherin, anti-N-cadherin, and anti-vimentin antibodies from BD Biosciences; anti-β-actin and anti-vinculin antibodies from Sigma; anti-GFP antibody from Neuro Mab; and anti-Snail antibodies from Cell Signaling. Rhodamine-conjugated phalloidin was obtained from Dojindo.

Clinical samples for quantitative real-time PCR

Cancer tissues were obtained with informed consent and approval of the ethics committee of Nagoya University. Details of patients’ samples are described in Supplementary Table S1.

siRNA screening

Transcription factors that exhibited increased expression in ovarian cancer cells were selected from data derived from experiments carried out by Welsh and colleagues and Lancaster and colleagues deposited in Oncomine (31–33). Forty-eight transcription factors were manually selected and 2 siRNAs targeting each gene were transfected to SKOV3 cells cultured in 24-well plates. Seventy-two hours later, luciferase activities were determined in triplicate, and 3 independent experiments were carried out. To measure luciferase activity in the absence of ALX1 expression, SKOV3 cells were transfected with control or ALX1 siRNA together with pRTK-Luc and pGL4-Snail/promoter. Seventy-two hours later, luciferase activities were measured.

Statistical analysis

Statistical analysis for cell invasion, colony formation, and tumor formation in mice was conducted using the Student t test. P < 0.05 was considered statistically significant. The association between ALX1 expression and tumor stage was determined by χ² test. The correlation between ALX1 and Snail expression or ALX1 and Slug expression in cancer tissues was determined by Pearson correlation coefficient.

Results

Depletion of ALX1 induces reversion of EMT

To search for novel transcription factors associated with EMT and aggressive characteristics in ovarian cancer cells, we conducted a limited siRNA screen. We manually selected 48 transcription factors that were previously reported to exhibit increased expression in ovarian cancer (32, 33). SKOV3 cells were transfected with siRNAs targeting these transcription factors and changes in cellular morphology were examined. We predicted that the depletion of genes relevant to EMT would induce an epithelial morphology with tight cell–cell adhesion in SKOV3 cells. In this screen, we discovered that the transfection of 2 different ALX1-targeting siRNAs induced morphologic changes in SKOV3 cells. Thus, we decided to investigate roles for ALX1 in the induction of EMT and the acquisition of aggressive characteristics in ovarian cancer cells.

We first evaluated the expression levels of ALX1 mRNA in ovarian cancer cell lines using quantitative RT-PCR analysis. The expression of ALX1 mRNA was greatest in the ES-2, HEY, and SKOV3 cells (Fig. 1A). Interestingly, the expression level of ALX1 mRNA was related with the malignant characteristics of ovarian cancer cells. ES-2, HEY, and SKOV3 cells were more invasive than other ovarian cancer cell lines and were able to grow under anchorage-independent conditions (Supplementary Fig. S1). To determine the expression of ALX1 in ovarian cancer, we conducted in situ hybridization using tissue microarrays. We found that strong expression of ALX1 mRNA was observed in some cancer tissues, and the expression was correlated with tumor stage (Fig. 1B). We then examined the effects of ALX1 depletion in the cell lines with high ALX1 expression. ALX1 depletion induced the recovery of cell–cell...
adhesions and the cellular morphology became more like that of epithelial cells (Fig. 1C). Immunostaining analysis revealed that knocking down ALX1 expression induced a clear recovery of E-cadherin localization in HEY and SKOV3 cells but not in ES-2 cells (Fig. 1C). We speculated that the cells had undergone a reversion of EMT by ALX1 depletion in HEY and SKOV3 cells. To test this hypothesis, the expression levels of epithelial and mesenchymal markers were examined. ALX1 mRNA expression was sufficiently suppressed by both siRNAs (Fig. 1D). The upregulation of E-cadherin and the downregulation of N-cadherin and vimentin were detected at both the mRNA and the protein levels in HEY and SKOV3 cells (Fig. 1D and E). However, ES-2 cells did not show any changes in expression of these markers by ALX1 knockdown (Fig. 1D and E). We carried out rescue experiment to exclude the possibility of off-target effects of siRNAs. SKOV3 cells that constitutively expressed GFP-ALX1 or siRNA-resistant GFP-ALX1 (ALX-Res#2) were established by retrovirus infection. ALX1 siRNA effectively ablated exogenously expressed wild-type GFP-ALX1 but not siRNA-resistant GFP-ALX1 (Supplementary Fig. S2). Changes in expression of the marker proteins were not observed in cells that expressed siRNA-resistant GFP-ALX1 (Supplementary Fig. S2). These results indicate that ALX1 is crucial for SKOV3 and HEY cells to maintain mesenchymal characteristics, but ES-2 cells may have additional factors that prevent cells from undergoing reversion of EMT by ALX1 depletion.

ALX1 regulates cell invasion and anchorage-independent growth

EMT is associated with malignant properties, such as invasion and anchorage-independent growth (35, 36). We tested whether ALX1 was required for these properties in ovarian cancer cells. To assess cell invasiveness, we used Matrigel-coated Boyden chambers. The invasion of HEY, SKOV3, and ES-2 cells was significantly suppressed by ALX1 knockdown (Fig. 2A). The reduced invasiveness by ALX1 depletion is not due to the impaired viability of cells because cell proliferation was not inhibited by ALX1 siRNA transfection (Supplementary Fig. S3). We next assessed anchorage-independent cell growth in the ALX1-depleted cells. As shown in Fig. 2B, the silencing of ALX1 expression suppressed colony formation...
in soft agar. To test whether the inhibition of ALX1 expression affected cancer cell growth in vivo, we generated SKOV3 cells that constitutively expressed short hairpin RNA (shRNA) targeting either luciferase (shCtrl) or ALX1 (shALX1). ALX1 mRNA was reduced and E-cadherin expression was induced in shALX1 cells (Fig. 2C). shCtrl and shALX1 cells were subcutaneously injected to the femoral area of nude mice and tumor formation was examined. Both cell lines formed 6 subcutaneous tumors of 7 injected sites. The tumor formation of shALX1 was suppressed compared with the tumor formation of shCtrl cells (Fig. 2D). Mice were sacrificed 36 days after tumor injection, and the tumor weight was determined. The average tumor weight of shALX1 cells was significantly reduced compared with that of shCtrl cells (Fig. 2E).

**Exogenous expression of ALX1 confers malignant phenotype.**

We next determined whether the exogenous expression of ALX1 confers a more malignant phenotype to ovarian cancer cells. In addition to a homeodomain in the central region of the protein, ALX1 has a conserved amino acid stretch known as the aristatless domain or OAR domain (Fig. 3A). We generated SKOV3 cells that constitutively expressed GFP (GFP), GFP-tagged full-length ALX1 (FL), or GFP-tagged ALX1 lacking either the homeodomain (Δhomeo) or the OAR domain (ΔOAR). FL and ΔOAR localized to the nucleus, but Δhomeo diffusely localized to both the cytoplasm and the nucleus (Fig. 3B). FL and ΔOAR SKOV3 cells exhibited more elongated and spindle-like shapes than the GFP and Δhomeo SKOV3 cells (Fig. 3C). In addition, the expression of N-cadherin and vimentin was upregulated in FL and ΔOAR SKOV3 cells but not in GFP and Δhomeo SKOV3 cells (Fig. 3C). In addition, the expression of N-cadherin and vimentin was upregulated in FL and ΔOAR SKOV3 cells but not in GFP and Δhomeo SKOV3 cells (Fig. 3C). We next assessed the effects of ALX1 expression on invasion and anchorage-independent growth. Both FL and ΔOAR SKOV3 cells were significantly more invasive than the GFP and Δhomeo SKOV3 cells (Fig. 3D). Furthermore, expression of FL and ΔOAR clearly promoted the anchorage-independent growth of SKOV3 cells (Fig. 3E). Promotion of EMT, anchorage-independent growth, and invasion by ALX1
Expression was also observed using additional ovarian cancer cell line, NOS3 (Supplementary Fig. S4). These results show that ALX1 is associated with aggressive phenotype of ovarian cancer cells.

Exogenous expression of ALX1 in nontumorigenic cells induces EMT

To further extend our analysis of the role of ALX1 in EMT, we determined whether ALX1 can induce EMT in nontumorigenic epithelial cells. MCF10A cells are nontransformed mammary epithelial cells characterized by well-organized cell–cell junctions. We generated GFP, FL, Δhomeo, and ΔOAR MCF10A cells by retroviral infection. Both GFP and Δhomeo MCF10A cells maintained a cobblestone-like morphology that was similar to parental cells (Fig. 4A). In contrast, FL and ΔOAR MCF10A cells appeared elongated and the cell–cell junctions were disrupted (Fig. 4A). E-cadherin was localized along the cell–cell junctions in GFP and Δhomeo MCF10A cells but was absent in FL and ΔOAR MCF10A cells (Fig. 4B). Remodeling of the actin cytoskeleton is often observed during EMT. Both GFP and Δhomeo MCF10A cells primarily exhibited cortical F-actin formation along cell–cell junctions, whereas the clear organization of actin stress fibers across the cell was observed in the FL and ΔOAR MCF10A cells (Fig. 4C). Consistent with the observed changes in actin cytoskeleton organization, the formation of focal adhesions was evident in FL and ΔOAR MCF10A cells (Fig. 4C). ALX1-induced EMT was further confirmed by immunoblot analysis. FL and ΔOAR MCF10A cells exhibited a decrease in E-cadherin expression that was concomitant with an increase in the expression of both N-cadherin and vimentin (Fig. 4D). The expression of ALX1 also promoted migration and invasion of MCF10A cells (Fig. 4E and F).

ALX1 regulates Snail expression

To gain further insight into the molecular mechanisms by which ALX1 regulates EMT and malignant conversion, we aimed to identify transcription factors whose expression is regulated by ALX1. RNA was extracted from SKOV3, HEY, and ES-2 cells that had been transfected with ALX1-targeting siRNA, and the mRNA expression of EMT-related transcription factors was examined by RT-PCR. Among the transcription factors examined, Snail mRNA was decreased by the knockdown of ALX1 in SKOV3 and HEY cells but not in ES-2 cells (Fig. 5A). The reduced expression of Snail protein in HEY and SKOV3 cells transfected with ALX1 siRNA was verified by immunoblot (Fig. 5B). Snail was localized in the nucleus of
SKOV3 cells that were transfected with control siRNA (Fig. 5C). ALX1 knockdown suppressed nuclear Snail expression, which was concomitant with an increase in E-cadherin expression along cell–cell junctions (Fig. 5C). We next determined whether the exogenous expression of ALX1 upregulates Snail expression in SKOV3 and MCF10A cells. Whole-cell extracts of GFP, FL, Δhomeo, and ΔOAR cells were subjected to immunoblot analysis and Snail expression was detected. As shown in Fig. 5D, Snail expression was increased in FL and ΔOAR cells compared with GFP and Δhomeo cells. We also conducted luciferase assays to determine whether Snail promoter activity was regulated by ALX1 expression. 293T cells were transiently cotransfected with full-length ALX1, and a reporter construct in which the human Snail promoter region was cloned upstream of firefly luciferase (pGL4-Snail/promoter). Exogenous expression of ALX1 increased Snail promoter activity approximately 3.3-fold (Fig. 5E). In addition, the transfection of ALX1 siRNA into SKOV3 cells decreased promoter activity 40% to 50% compared with that of luciferase siRNA (Fig. 5F). These results indicate that ALX1 induces the transcription of the Snail gene. Finally, we examined whether ALX1 expression was related with Snail expression in ovarian cancer. The expression levels of ALX1 and Snail mRNA were evaluated in 19 ovarian cancer tissues by quantitative RT-PCR. As shown in figure 5G, we observed a significant correlation between ALX1 and Snail expression. However, the expression of Slug, a homolog of Snail, did not correlate with ALX1 expression (Fig. 5G).

Snail is required for ALX1-mediated EMT and cell invasion

If Snail expression is crucial for the ALX1-mediated EMT, then the depletion of Snail is expected to reverse the morphologic changes induced by ALX1 expression. To test this possibility, MCF10A cells that constitutively expressed GFP-ALX1 were transfected with Snail siRNA and changes in the expression of EMT-related proteins were examined. Immunofluorescence analysis showed that Snail depletion clearly restored E-cadherin localization to the cell–cell junctions (Fig. 6A).
E-cadherin expression and the decrease in both N-cadherin and vimentin expression were confirmed by immunoblot analysis in Snail knockdown cells (Fig. 6B). We next examined whether Snail expression was critical for ALX1-mediated EMT and cell invasion in SKOV3 cells. Similar to the results obtained from MCF10A cells, silencing Snail expression in ALX1-expressing SKOV3 cells restored epithelial properties, as indicated by the increased expression of epithelial markers (Fig. 6C). In addition, the invasive phenotype of ALX1-expressing SKOV3 cells induced an increase in E-cadherin expression and a decrease in both N-cadherin and vimentin expression, whereas ALX1 depletion in GFP-Snail SKOV3 cells did not induce changes in the expression of these marker proteins (Fig. 6E). We next assessed the invasion of GFP-Snail SKOV3 cells in the absence of ALX1 expression. As shown in figure 6F, ALX1 depletion did not suppress invasion in GFP-Snail SKOV3 cells. These results indicate that the ALX1-mediated promotion of EMT and cell invasion is dependent on Snail expression.

**Discussion**

Accumulating evidence indicates that EMT-associated transcription factors confer cancer cells with malignant...
characteristics, such as invasion, metastasis, and resistance to chemotherapy (15). In this study, we determined that the exogenous expression of ALX1 in SKOV3 cells induced mesenchymal characteristics and promoted cell invasion and colony formation in soft agar. The depletion of ALX1 induced morphologic changes and suppressed anchorage-independent growth and cell invasion in HEY, SKOV3, and ES-2 cells. In addition, clear reversion of EMT was observed by ALX1 depletion in HEY and SKOV3 cells. Furthermore, the silencing of ALX1 expression in SKOV3 cells significantly suppressed tumor formation in mice. Collectively, these results indicate that ALX1 contributes to the promotion of EMT and the acquisition of malignant characteristics in ovarian cancer cells. However, ALX1-mediated EMT appears to be dependent on the cellular context because ALX1 depletion did not induce reversion of EMT in ES-2 cells. We also found that Snail expression was positively regulated by ALX1. Depletion of Snail inhibited ALX1-mediated EMT and cell invasion, indicating that the ALX1/Snail axis is a novel pathway that contributes to the malignant conversion of ovarian cancer cells. A previous immunohistochemical analysis of ovarian cancer tissue showed that Snail expression was high in metastatic lesions and that Snail expression correlates with tumor stage (37). Interestingly, we found that the expression of Snail and ALX1 was correlated in ovarian cancer tissues. These results suggest that ALX1 may regulate Snail expression in ovarian cancer, thereby promoting malignant conversion. In addition to ovarian cancer, Snail expression is associated with a poor prognosis and the recurrence of other tumors (16); thus, ALX1 may promote tumor progression in cancers other than ovarian cancer.
The exogenous expression of ALX1 induced EMT not only in cancer cells but also in nontumorigenic MCF10A mammary epithelial cells. The expression of ALX1 in MCF10A cells induced the expression of mesenchymal markers and the reorganization of the cytoskeleton, which are changes associated with EMT. The ability of ALX1 to induce EMT in nontumorigenic epithelial cells suggests that ALX1 may possess crucial roles in promoting EMT during development. Homozygous deletion of ALX1 gene in humans is associated with the severe disruption of early craniofacial development (29). ALX1 knockout mice exhibit defective neural tube closure and limb girdle development (28, 38). These studies clearly show that ALX1 plays an essential role in mammalian development, but how ALX1 controls embryonic development remains largely unknown. Recent studies have shown that ALX1 is a part of a gene regulatory network that is required for the development of sea urchins (39). ALX1 regulates the expression of genes, such as Twist, that induce EMT in primary mesenchyme cells (40). Therefore, ALX1 may contribute to the induction of EMT during the mammalian development, thereby promoting limb girdle and craniofacial formation.

The expression of EMT-associated transcription factors is regulated by integrated and complicated regulatory systems. Previous studies have elucidated some of the upstream regulators of ALX1 expression during development. A heterodimer composed of Pbx1 and Emx2, both of which are homeoproteins, binds directly to the conserved upstream sequence of the ALX1 gene (41). During sea urchin development, the activation of β-catenin induces ALX1 expression (27), and the phosphorylation of the Ets1 transcription factor by mitogen-activated protein kinase (MAPK) signaling maintains ALX1 expression (39). These findings implicate the existence of multiple regulatory mechanisms to control ALX1 expression. Further investigation may reveal that these transcription factors promote EMT and malignant conversion of cancer cells by regulating ALX1 expression.

In summary, we have identified ALX1 as a novel regulator of EMT and cell invasion. In addition, the induction of Snail expression was shown to be critical for the ALX1-mediated promotion of EMT and cell invasion. ALX3 and ALX4 are both ALX1 homologues that are required for craniofacial development in humans (42, 43). Whether ALX3 and ALX4 are associated with EMT and cancer progression remains unknown. The elucidation of the cellular functions of these ALX family members will provide additional information that may greatly improve our understanding of the regulatory networks governing EMT and cancer progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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