Original Paper

Id-1, a Protein Repressed by miR-29b, Facilitates the TGFβ1-Induced Epithelial-Mesenchymal Transition in Human Ovarian Cancer Cells

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Key Words

Inhibitor of DNA binding 1 (Id-1) • Epithelial-Mesenchymal Transition (EMT) • Transforming Growth Factor Beta 1 (TGFβ1) • Micro-RNAs • Ovarian Cancer

Abstract

Background: Transforming growth factor beta 1 (TGFβ1) can induce epithelial-mesenchymal transition (EMT) in various human cancers, but the complex mechanisms underlying this have not been fully elucidated. Inhibitor of DNA binding 1 (Id-1) has been identified as a novel marker of ovarian cancer progression. This study aims to investigate the role of Id-1 in TGFβ1-induced EMT in human ovarian cancer cells. **Methods:** Ovarian cancer cells expressing or not expressing Id-1 were incubated with TGFβ1. Changes in the EMT markers E-cadherin, vimentin, N-cadherin, Id-1, and miR-29b were detected using western blotting and qPCR analyses. Wound healing, transwell migration, and invasion assays were performed in cells where Id-1 was either knocked down or overexpressed. The effects of transfecting miR-29b mimics and inhibitors on Id-1 mRNA and protein expression were assessed. The interaction between miR-29b and Id-1 was confirmed using a luciferase reporter assay. **Results:** Id-1 expression was increased and miR-29b expression was repressed in TGFβ1-responsive ovarian cancer cells. Id-1 overexpression increases and Id-1 knockdown decreases cell migration and invasion capacities. Id-1 silencing leads to a partial blocking of TGFβ1-induced EMT. miR-29b negatively regulates Id-1 expression. Direct binding of miR-29b to the 3'UTR region of Id-1 was confirmed using a luciferase reporter assay. **Conclusion:** Id-1, a protein repressed by miR-29b, facilitates TGFβ1-induced EMT in human ovarian cancer cells and represents a promising therapeutic target for treating ovarian cancer.

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Introduction

Epithelial ovarian cancer (EOC) accounts for 25% of all malignancies affecting the female genital tract and is the most lethal gynecological malignancy. It is estimated that there were 22,240 new ovarian cancer cases and 14,030 deaths in the United States in 2013 [1]. Despite the use of aggressive treatment, most EOC patients develop recurrent cancer, and cancerous metastasis is one of the leading causes of death [2]. Emerging evidence suggests that the epithelial-mesenchymal transition (EMT) plays a crucial role in the progression of EOC by increasing cancer cell motility, chemo-resistance, and cancer stem cell characteristics [3]. Therefore, targeting EMT in EOC might be a feasible and effective therapeutic option.

EMT refers to the depolarization and dedifferentiation of epithelial cells to mesenchymal cells. This process is characterized by the loss of epithelial features such as cellular polarity and inter-cellular interactions and by the acquisition of mesenchymal characteristics via multiple approaches including rearrangement of the cytoskeleton, alteration of adhesion molecule expression, and modification of cellular structure and morphology [4, 5]. EMT has been confirmed to be involved in physiological and pathological processes, including normal embryonic development, fibrosis, and cancer metastasis [6]. Because increased motility occurs during this transition, EMT is currently recognized as an important event and the initiating step for tumor invasion and metastasis. A variety of cytokines and growth factors are induced during EMT, which further stimulates the EMT process and promotes cell migration and invasion; these factors include TGF β (transforming growth factor β), Wnt, Notch ligands, ILEI (interleukin-like EMT-inducer), EGF (epidermal growth factor), PDGF (platelet-derived growth factor), and HGF (hepatocyte growth factor) [7-10].

Although several growth factors participate in EMT, TGF β is the most studied. The multipotent cytokine TGF β exhibits three isoforms: TGF β 1, 2, and 3. Among the three isoforms, TGF β 1 is the most abundant in most tissues and has been found to play an essential role in the induction of EMT, not only during embryogenesis but also during cancer progression [11-13]. Interestingly, TGF β has been shown to play diverse and contradictory roles during various stages of carcinogenesis. In early stage cancers, TGF β serves as a tumor suppressor by inducing growth arrest and promoting apoptosis. In advanced cancers, TGF β expression is often upregulated, consequently promoting cancer progression through the induction of EMT, during which tumor cells become more invasive and metastatic [13, 14]. Although TGF β was first found to induce EMT almost 20 years ago, the complex mechanisms underlying this induction have not yet been fully elucidated [15]. In addition to the canonical Smad signaling pathway and several non-Smad signaling pathways (including the MAPK, PI3K, and GTPase pathways) that have been well-studied and widely acknowledged, additional non-canonical pathways and levels of regulation are still being discovered; these phenomena include the involvement of microRNAs in regulating relevant gene expression and their interactions with various signaling pathways [16-18].

Id-1 is a member of the Id (inhibitor of differentiation or DNA binding) protein family, which belongs to the HLH (helix-loop-helix) family of transcription factors. In contrast to other bHLH (basic HLH) members, Ids lack basic DNA-binding domains and function as dominant negative regulators by forming heterodimers with bHLH proteins and preventing them from binding to DNA in many biological processes, including cell differentiation, cell senescence, neurogenesis, apoptosis, and angiogenesis [19-22]. Upregulation of Id-1 has been found in ovarian cancer and is associated with dedifferentiated, proliferating, and invasive cell phenotypes, as well as advanced tumor stage and poor prognosis [23, 24]. It has therefore been suggested that Id-1 may play a crucial role not only in tumorigenesis but also in the progression and metastasis of ovarian cancer. Recent studies have indicated the involvement of Id-1 in EMT regulation [25-27]. Hu and colleagues found that increased Id-1 expression was associated with advanced bladder cancer stage and grade [25]. In addition, an increase in Id-1 expression was also correlated with decreased membranous expression in bladder tumors of E-cadherin and β -catenin, both of which are critical epithelial and mesenchymal biomarkers. *In vitro* studies further showed that the inactivation of Id-1

in bladder cancer cells shifted the cellular morphology from a fibroblastic to epithelial appearance, whereas the overexpression of Id-1 could lead to the acquisition of a fibroblastic spindle cell phenotype and the loss of intercellular contacts. Together, these results suggest that Id-1 might play a role in tumor progression and EMT activation in bladder cancer. Other researchers found that immortalized epithelial cells expressed higher endogenous levels of Id-1 than normal cells [26] and that ectopic Id-1 expression inhibited the differentiation of immortalized esophageal epithelial cells and promoted cadherin switching, as well as increased adhesiveness to the extracellular matrix, motility, migratory potential, and invasiveness. Thus, it has been speculated that Id-1 might induce cell invasiveness in immortalized epithelial cells, possibly by regulating cadherin switching and rho GTPases. However, the specific molecular signaling responsible for the role of Id-1 in EMT, especially under the unique condition of TGF β 1-induced EMT in ovarian cancer, remains largely unknown. Therefore, we aimed to explore the role of Id-1 using our model of EMT in ovarian cancer cells.

Micro-RNAs are a class of non-coding RNAs that are 20 to 25 nucleotides in length. Thus far, more than 1,000 micro-RNAs have been identified in mammalian cells [28]. Recent studies have indicated that micro-RNAs can regulate gene expression at the post-transcriptional level through decreased translation of the target mRNA, increased degradation of the target mRNA, or both [29, 30]. By binding to the 3'UTR of target mRNA, micro-RNAs interact directly with target genes and thereby downregulate their expression. Micro-RNAs also play pivotal roles in cell growth, differentiation, apoptosis, and tumorigenesis [31-34]. miR-29b, a member of the micro-RNA29 family, has been shown to participate in both the onset and progression of various malignant tumors, including ovarian cancer [35-39]. For example, a clinical study demonstrated that miR-29b was downregulated in a significant proportion of ovarian serous carcinoma samples and that high miR-29b expression was associated with reduced disease-free survival [39]. More recently, Dai F and coauthors demonstrated that a chimeric model comprising a MUC1 aptamer and miR-29b could efficiently deliver miR-29b into the ovarian cancer cell line OVACAR-3, which in turn induced apoptosis. The authors further discovered that the apoptotic role of miR-29b in ovarian cancer cells was mediated through a hypomethylation machinery-mediated upregulation of PTEN expression [40]. These studies collectively confirmed the involvement of miR-29b in ovarian cancer development and progression. miR-29b is also decreased in TGF β 1-induced fibrosis [41-43]. However, to the best of our knowledge, the specific role of miR-29b in the TGF β 1-induced EMT in ovarian cancer and the mechanisms underlying its effects remain unknown. Because EMT is of particular significance as a marker of tumor invasion and metastasis and because TGF β 1 treatment represents a classical induction approach for *in vitro* EMT research, we believe that elaborating both the specific roles of miR-29b in TGF β 1-induced EMT models of ovarian cancer and the latent molecular mechanisms will enlarge our theoretical understanding of human ovarian cancer and provide future clinical approaches to treating this disease.

Materials and Methods

Cell culture and TGF β 1 treatment

Human ovarian cancer cell lines SKOV3 (obtained from ATCC, Manassas, VA, USA) and 3AO (purchased from the Chinese Academy of Sciences Type Culture Collection, Shanghai, China) were maintained in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum at 37°C under a humidified 5% CO₂ atmosphere. In the TGF β 1 (Sigma Aldrich, St. Louis, MO) treatment, the cells were serum starved overnight and treated with 10 ng/ml TGF β 1 for 48 hours. The medium containing TGF β 1 was replaced every 24 hours.

Plasmid and transient transfection

The human Id-1 expression vector pcDNA3 hId1 (Addgene, #16061) was kindly provided by Robert Benezra. Ovarian cancer cells were seeded into 6-well plates until 80% confluent and transiently transfected with pcDNA3 hId1 or empty vector (pcDNA3) as a control using X-treme GENE HP DNA Transfection Reagent

Table 1. Primer and siRNA sequences

Genes	Primer Sequence (5'-3')	Experimental use
Id-1	F:CTTCGGGCTTCCACCTCATT R:GACAGACAGCGCACCTC	Real-time PCR
E-cadherin	F:GCTGCTCTTGCTGTTTCTTCG R:CCGCCTCCTTCTTCATCATAG	Real-time PCR
N-cadherin	F:GCCCCCTCAAGTGTACCTCAA R:AGCCGAGTGATGGTCCAATTT	Real-time PCR
Vimentin	F:AAGTTTGCTGACCTCTCTGAGGCT R:CTTCATTTACGCATCTGGCGTT	Real-time PCR
Beta-actin	F:TCCCTGGAGAAGAGCTACGA R:AGCACTGTGTGGCGTACAG	Real-time PCR
Id-1 siRNA	CATGAACGGCTGTTACTCA	RNA interference
NC siRNA	UUCUCCGAACGUGUCACGUTT	RNA interference

(Roche, Indianapolis, IN, USA) following the manufacturer's protocol. After 48 hours of transfection, the cells were harvested for further study.

siRNA and transient transfection

Human Id-1 siRNA and negative control (NC) siRNA were purchased from GenePharma (Shanghai, China). Ovarian cancer cells were seeded into 6-well plates until they reached 30%–50% confluence. Id-1 siRNA was transiently transfected using the X-treme GENE siRNA Transfection Reagent (Roche, Indianapolis, IN, USA). After 48 hours of siRNA transfection, the cells were harvested for further studies. For experiments in which cells were exposed to both TGF β 1 and siRNA, the cells were first transfected with Id-1 siRNA or NC siRNA for 24 hours and then incubated with TGF β 1 for 48 hours. The sequences of Id-1 siRNA and NC siRNA are listed in Table 1.

microRNA and transient transfection

miR-29b mimics, control mimics, miR-29b inhibitors, and control inhibitors were purchased from RiboBio (Guangzhou, China). SKOV3 cells were seeded into 6-well plates until 50%–60% confluent and then transiently transfected with 60 nM control or miR-29b mimics or with 120 nM control or miR-29b inhibitors using the X-treme GENE siRNA Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. After 48 hours of miRNA transfection, the cells were harvested for further study.

Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. For mRNA detection, first-strand cDNA was synthesized using a PrimeScript RT reagent kit (Perfect Real Time; Takara, Dalian, China). Quantitative real-time PCR was performed using a SYBR Premix Ex Taq™ II kit (Takara, Dalian, China) on a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). β -Actin was used as an internal control to normalize the results. The primers used are listed in Table 1. For miRNA detection, miR-29b levels were determined using a TaqMan microRNA kit (Applied Biosystems) and normalized to small nuclear RNA (Rnu6), which served as a control; the data were expressed as the log 2 fold change in respective miR/U6 snRNA levels. Primers for miR-29b and U6 reverse transcription and amplification were designed by and purchased from RiboBio Co., Ltd. (Guangzhou, China).

Western blot analysis

Cell lysates were collected using mammalian protein extraction reagent (Pierce, Rockford, IL, USA) containing protease inhibitors (Roche, Indianapolis, IN, USA). The protein concentrations in each sample were determined using the BCA-200 protein assay kit (Pierce, Rockford, IL, USA). The proteins were resolved on 12% (for Id-1 detection) or 10% (for other protein detection) SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were then blocked using blocking buffer (5% non-fat milk in TBST) and incubated overnight at 4°C with rabbit antihuman Id-1 (SC-488, Santa Cruz, CA, USA), E-cadherin (R868, Bioworld, MN, USA), N-cadherin (13769-1-AP, Proteintech™, Glostrup, Denmark), vimentin (#5741S, CST, MA, USA), and mouse antihuman β -actin (#3700S, CST, MA, USA) at dilutions of

1:500, 1:600, 1:500, 1:800, and 1:2000, respectively. After washing with TBST, the blots were visualized using peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG and ECL reagents (Pierce, Rockford, IL, USA).

Wound healing assay

Ovarian cancer cells were seeded into 6-well plates until they reached 90%–95% confluence. Wounds of similar size were induced on the cell monolayer using a sterile yellow pipette tip, after which the medium containing floating cells was carefully removed. Prewarmed serum-free medium (2 ml) was then added. The speed of wound closure was monitored and photographed at intervals using a phase-contrast microscope under 100x magnification.

Transwell migration and invasion assay

In vitro cell migration assays were performed using Transwell chambers (8 μm pore size; Costar). Upon reaching 75%–80% confluence, cells were serum starved overnight. After detachment using trypsin and washing with PBS, the cells were resuspended in serum-free medium, and a total of 3×10^5 cells in 100 μl serum-free medium was added to the upper chamber. Complete medium was added to the bottom chambers. Cells that had not migrated were removed from the upper face of the filters using cotton swabs, and cells that had migrated were fixed using 5% glutaraldehyde solution and stained using a 0.5% solution of Toluidine Blue in 2% sodium carbonate. Images of five random fields ($\times 400$) on each membrane were captured, and the number of migratory cells was counted; the mean values of three duplicate assays for each experimental condition were used for statistical analysis. Similar inserts coated with Matrigel were used to determine invasive potential in the invasion assay.

Luciferase reporter assay

The *Id-1* 3'UTR containing the predicted *miR-29b* target sequence was amplified from genomic DNA (SKOV3 cells) and cloned into the pGL3 firefly luciferase control vector (Promega, Madison, WI) at the *XhoI* restriction site immediately downstream of the luciferase reporter gene. To generate an *Id-1* 3'UTR with a mutant target sequence, transversion mutations of 7 nucleotides were constructed at sites complementary to the *miR-29b* seed region, as shown in Fig. 5D. Post-transcriptional inhibition of the luciferase reporter gene by *miR-29b* was assayed in SKOV3 cells. Briefly, ovarian cancer cells were seeded into 24-well plates and cultured until they reached 70%–80% confluence. The cells were then co-transfected with either *miR-29b* or control mimics at a final concentration of 120 nM together with 200 ng of a pGL3 reporter construct containing wild type or *miR-29b* site-mutated *Id-1* 3'UTR using the X-treme GENE HP DNA Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Transfections were performed in quadruplicate. The relative firefly luciferase activity (normalized to *Renilla* luciferase activity) was measured 48 hours post-transfection using a dual-luciferase reporter gene assay system (Promega, Madison, WI, USA), and results were plotted as the percentage change over the respective control.

Statistical analysis

All experiments were performed at least in triplicate, and each experiment was independently performed at least 3 times. Data are presented as the means $\pm$ standard deviation (SD) and were analyzed using SPSS 19.0 and GraphPad Prism 5 software. Statistical significance was assessed using the two-tailed unpaired Student's *t*-test. Differences were considered statistically significant when the *P* value was <0.05 .

Results

TGFβ1 induces EMT in ovarian cancer cells

In preliminary experiments, we tested various TGFβ1 concentrations and incubation durations for their ability to induce EMT in SKOV3 and 3AO cells; based on these experiments, we determined the dose of 10 ng/ml and the duration of 48 hours as appropriate conditions for EMT stimulation by TGFβ1. To compare the stimulation results, we examined morphological changes; we also compared the expression of EMT markers such as E-cadherin, N-cadherin, and vimentin using qPCR and western blotting analyses (Fig. 1). Both SKOV3 and 3AO

Fig. 1. TGF β 1 induces EMT in ovarian cancer cells. The human ovarian cancer cell lines SKOV3 and 3AO were incubated with 10 ng/ml TGF β 1 for 48 hours. (A) Compared with cells cultured under basal conditions, TGF β 1-treated SKOV3 and 3AO cells lost their cobblestone-like shape and adopted a fibroblast-like, spindle-shaped morphology. Red arrows indicate cells with typical phenotypic changes. Scale bar: 100 μ m. Both qPCR (B) and western blotting (C) revealed that TGF β 1 significantly downregulated the expression of the epithelial marker E-cadherin and upregulated the expression of the mesenchymal markers N-cadherin and vimentin. Data are presented as means $\pm$ SEM, n=3. * p<0.05 versus control.

Fig. 2. Id-1 expression is sharply increased in a TGF β 1-induced EMT model of ovarian cancer. SKOV3 and 3AO cells were incubated with 10 ng/ml TGF β 1 for 48 hours to induce EMT. Compared with cells cultured under basal conditions, TGF β 1-treated ovarian cancer cells exhibited significant increases in Id-1 expression in both qPCR (A) and western blotting (B) experiments. Data are presented as means $\pm$ SEM, n=3. * p<0.05 versus control.

cells that were cultured in basal media exhibited a classical epithelial morphology with cobblestone-like shapes. After incubation with 10 ng/ml TGF β 1 for 48 hours, the cells adopted a more fibroblast-like, elongated, and spindle-shaped morphology (Fig. 1A). Both qPCR (Fig. 1B) and western blotting analyses (Fig. 1C) showed that TGF β 1 treatment sharply decreased the expression of the epithelial marker E-cadherin and increased the expression of the mesenchymal markers vimentin and N-cadherin. Together, these results confirmed that incubation with 10 ng/ml TGF β 1 for 48 hours successfully induced EMT in both SKOV3 and 3AO cells.

Id-1 expression is sharply increased in TGF β 1-induced EMT

To explore whether Id-1 is involved in TGF β 1-induced EMT in human ovarian cancer cells, we first attempted to determine whether the expression of Id-1 was different after

Fig. 3. Id-1 expression is positively correlated with ovarian cancer cell motility. (A) In a transwell migration assay, plasmid vector-mediated Id-1 overexpression in 3AO cells led to an approximately doubling in migrating capacity. (B) In a transwell migration assay, siRNA-mediated Id-1 knockdown in SKOV3 cells decreased the cell migration capacity by approximately 50%. (C) In a transwell invasion assay, plasmid vector-mediated Id-1 overexpression in 3AO cells approximately increased the cell invading capacity twofold. (D) In a transwell invasion assay, siRNA-mediated Id-1 knockdown in SKOV3 cells decreased cell migration capacity by approximately 60%. (E) Representative images of wound healing show that plasmid vector-mediated Id-1 overexpression in 3AO cells increased the wound healing rate. (F) Representative images of wound healing show that siRNA-mediated Id-1 knockdown in SKOV3 cells slowed wound healing. The images of the transwell migration and invasion assays were photographed under 400x magnification, whereas the images of the wound healing assays were photographed under 100x magnification. Data are presented as means $\pm$ standard deviation of an individual set of experiments performed in triplicate.

expression in 3AO cells increased the wound healing rate. (F) Representative images of wound healing show that siRNA-mediated Id-1 knockdown in SKOV3 cells slowed wound healing. The images of the transwell migration and invasion assays were photographed under 400x magnification, whereas the images of the wound healing assays were photographed under 100x magnification. Data are presented as means $\pm$ standard deviation of an individual set of experiments performed in triplicate.

TGF β 1 treatment. Surprisingly, compared with the levels observed in SKOV3 and 3AO cells cultured in basal medium, Id-1 expression at both the mRNA and protein levels (determined using qPCR (Fig. 2A) and western blotting analysis (Fig. 2B), respectively) was significantly increased in cells lines incubated with TGF β 1, suggesting that Id-1 does play a role in TGF β 1-induced EMT of ovarian cancer cells.

Fig. 4. TGF β 1-mediated changes in EMT features are attenuated by Id-1 knockdown. (A) Phase contrast images of SKOV3 cells transiently transfected with Id-1 or control siRNAs followed by two days of culture in the presence or absence of TGF β 1. Scale bar: 100 μ m. (B) Western blotting analysis showing differences in epithelial and mesenchymal markers between cells treated with TGF β 1 alone and cells treated with both TGF β 1 and Id-1 siRNA.

Id-1 expression is positively correlated with ovarian cancer cell motility

Enhanced cellular motility, including migration and invasion abilities, is a unique feature of the mesenchymal phenotype acquired during the EMT process. Because incubation with TGF β 1 resulted in robust increases in Id-1 mRNA and protein levels in both SKOV3 and 3AO cells, we employed gene knock-out and knock-in techniques to test whether Id-1 expression is involved in motility changes in ovarian cancer cells, aiming to examine the specific role of Id-1 in TGF β 1-induced EMT (Fig. 3). The cell line SKOV3, which constitutively expresses high levels of Id-1, was transfected with Id-1 siRNA to knock down its endogenous Id-1 expression or with a scrambled siRNA as a control. The cell line 3AO, which constitutively expresses low levels of Id-1, was transfected with the human Id-1 expression vector pcDNA3 hId1 to overexpress Id-1 or with the pcDNA3 empty vector as a control. In a transwell migration assay, a higher percentage of high-Id-1-expressing cells (si-NC SKOV3 cells and pcDNA3 hId1 3AO cells) grew extensively inside the lower chamber compared to their low-Id-1-expressing counterparts (si-Id1 SKOV3 and pcDNA3 3AO cells) ($P < 0.05$); thus, the high-expressing cells exhibited stronger migration ability (Fig. 3A and 3B). A collagen invasion assay (Fig. 3C and 3D) generated results that were similar to those obtained using the transwell migration assay, suggesting that overexpression of Id-1 resulted in an enhanced invasion capacity, whereas knockdown of Id-1 led to a decrease in ovarian cancer invasion capacity ($P < 0.05$). Next, the cell migration rates of cell lines with lower Id-1 expression were shown to be significantly lower than cell lines with higher Id-1 expression, as evaluated using a wound-healing assay. As shown by the representative images presented in Fig. 3E and 3F, wound healing was slower in si-Id1 SKOV3 and pcDNA3 3AO cells than in si-NC SKOV3 and pcDNA3 hId1 3AO cells. These results demonstrate that Id-1 promotes the migration and invasion of ovarian cancer cells, possibly through the induction of EMT. In contrast, Id-1 inactivation inhibits the migration and invasion of ovarian cancer cells, perhaps through the reversal of EMT. In short, Id-1 plays an important role in mediating the motility of metastatic ovarian cancer cells.

Id-1 attenuation blocked TGFβ1-induced EMT in ovarian cancer cells

To further assess the effects of Id-1 on TGF β 1 activity, we transfected control and Id-1-targeting siRNAs into SKOV3 cells, where Id-1 expression is constitutively high, and looked for changes caused by Id-1 repression. Morphologically, Id-1 knockdown inhibited the mesenchymal transformation of SKOV3 cells induced by TGF β 1 (Fig. 4A). Specifically, ovarian cancer cells incubated with TGF β 1 exhibited elongated but narrow spindle shapes whereas

Fig. 5. Id-1 is negatively regulated by miR-29b post transcription. (A) qPCR results indicate that miR-29b expression is sharply decreased in TGF β 1-induced EMT in both SKOV3 and 3AO cells. (B) Micro-RNA mimic-mediated overexpression of miR-29b markedly repressed Id-1 at both the mRNA and protein level in SKOV3 cells. (C) Knockdown of miR-29b significantly increased Id-1 expression at both the mRNA and protein level in SKOV3 cells. (D) Schematic representation of various Id-1 3'UTR luciferase reporters used in the transfection experiments. (E) SKOV3 cells were co-transfected with either miR-29b or control mimics and 200 ng of a pGL3 reporter construct containing wild type or miR-29b site-mutated Id-1 3'UTR. The firefly luciferase activity normalized relative to *Renilla* luciferase activity was measured 48 hours post transfection. The results are plotted as a percentage change over the respective control. Data are presented as means $\pm$ SEM. * $p < 0.05$ versus control.

cells treated with both TGF β 1 and Id-1 siRNA manifested a more epithelial-like phenotype with typical cobblestone shapes. The effect of Id-1 knockdown on TGF β 1-induced changes in gene expression was examined using western blotting. As with the previous experiment, the combination of Id-1 knockdown and TGF β 1 treatment blunted the suppression of epithelial genes and upregulated the expression of mesenchymal genes compared to treatment with TGF β 1 alone (Fig. 4B). Collectively, these results show that Id-1 attenuation blocked the morphological and gene expression changes induced by TGF β 1 incubation in ovarian cancer cells.

Id-1 is negatively regulated by a microRNA, miR-29b, the expression of which is repressed in TGF β 1-induced EMT

We then explored the specific mechanism by which Id-1 is regulated. In the TGF β 1-mediated model of EMT in ovarian cancer cells, we tested changes in the expression of miR-29b and found that it was repressed sharply in both SKOV3 and 3AO cell lines (by approximately 70% and 85%, respectively, as assessed using qPCR (Fig. 5A)). Next, we employed micro-RNA mimics and inhibitors to specifically overexpress and knock down the endogenous expression of miR-29b in SKOV3 cells. As shown in Fig. 5B and 5C, Id-1 expression was significantly decreased by transfection with miR-29b mimics and was greatly increased by transfection with miR-29b inhibitors at both the mRNA and protein level. Therefore, miR-

29b negatively regulates Id-1 expression in ovarian cancer cells. Furthermore, we analyzed the 3'UTR sequence of Id-1 and the mature chain sequence of miR-29b, and the results indicated that the "seed region" in the miR-29b mature chain is fully complementary with and thus potentially binds to the Id-1 3'UTR sequence (Fig. 5D). This observation raises the possibility that miR-29b might negatively regulate Id-1 expression by directly binding to the Id-1 3'UTR sequence. Finally, a 3'UTR luciferase reporter assay confirmed that miR-29b directly binds to the Id-1 3'UTR. Briefly, SKOV3 cells were transfected with the miR-29b or control mimics plus a luciferase construct containing either the wild-type Id-1 3'UTR or a mutant Id-1 3'UTR (Fig. 5D). Only transfection of the wild-type Id-1 3'UTR significantly decreased ($P < 0.05$) luciferase expression. This suppressive effect of miR-29b was abolished by mutating the miR-29b site in the Id-1 3'UTR (Fig. 5E). Together, these results demonstrated that miR-29b directly binds to its complementary sequence motif in the Id-1 3'UTR, thus negatively regulating Id-1 expression.

Discussion

In the present study, we found that Id-1 expression is specifically upregulated in TGF β 1-responsive ovarian cancer cells and that it contributes significantly to the development of the EMT phenotype. The EMT is characterized by changes in morphology, the loss of intercellular junctions, increased motility, decreased proliferation, and alterations in gene expression [44]. Our study demonstrated that Id-1 knockdown inhibits these TGF β 1-induced changes. Similarly to our observations, knockdown of Id-1 is also found to inhibit TGF β 1-induced migration in other types of human cancer cells, such as prostate cancer cells [45, 46]. Taken together, these findings confirm the involvement of Id-1 in TGF β 1-mediated EMT. Moreover, dysregulation of Id-1 is frequently found in various human cancers, including ovarian cancer, prostate cancer, endometrial cancer, and breast cancer, and is associated with features of cancerous progression and metastasis [23, 46-48]. In our study, gene knock-in and knock-out experiments also demonstrated that the siRNA-mediated silencing of Id-1 leads to decreased migration and invasion capacities, while plasmid-mediated Id-1 overexpression results in enhanced migration and invasion capacities in ovarian cancer cells; both these results indicate that the expression of Id-1 is positively correlated with ovarian cancer cell motility and further corroborate the connection between Id-1 expression and EMT features in human ovarian cancer cells. Therefore, targeting Id-1 might represent a feasible and attractive option for the future clinical treatment and prevention of human ovarian cancer.

Recently, evidence is accumulating that indicates that micro-RNAs play a substantial role in carcinogenesis by altering the expression of oncogenes and/or tumor suppressors at the post-transcriptional level [49]. Our data further support this conclusion by indicating that decreased miR-29b expression with concomitant enhancement of Id-1 expression may partly explain the upregulation of Id-1 in ovarian cancer cells, further demonstrating the correlation between the progressive and metastatic potential of human ovarian cancer. By employing the Dual Luciferase Reporter Assay System, we confirmed that the "seed region" of miR-29b directly binds to the 3'UTR of Id-1 mRNA, thus specifying the mechanism by which miR-29b represses Id-1 at both the mRNA and protein level in our cell models of human ovarian cancer. Others have demonstrated that Id-1 interacts with miR-29b in human lung cancer cells [50]. Collectively, these findings confirm that miR-29b plays a significant role in cancer development and progression by directly interacting with and repressing the known oncogene Id-1. Moreover, miR-29b is dysregulated in several human cancers, including prostate cancer, non-small-cell lung cancer, and breast cancer [51-53]. In addition, several studies show that miR-29b plays a role in regulating the tumor micro-environment and in mediating cancer metastasis [54-57]. Together, the evidence above suggests the possibility that miR-29b might represent a novel target for preventing cancer metastasis in future clinical applications.

Regarding the interaction between TGFβ1 and miR-29b, recent studies have focused mainly on the role of miR-29b in TGFβ1-mediated fibrosis. For example, a recently published study demonstrated that miR-29 mediated TGFβ1-induced renal fibrosis by manipulating the expression of disintegrin metalloproteases (ADAMs) [41]. In a study comparing the levels of several micro-RNAs in the urine of forty-three IgA nephropathy patients and thirteen healthy controls, it was discovered that urinary miR-29b levels are correlated with proteinuria and renal function and are significantly correlated with urinary Smad3 levels; this observation suggests that miR-29b might be regulated by the TGFβ1/SMAD3 pathway, and thus, miR-29b might have essential functions in the development of progressive renal fibrosis in IgA nephropathy [58]. However, no information is presently available on whether miR-29b is associated with TGFβ1-induced cancerous EMT. In our study, we observed a marked repression of miR-29b in TGFβ1-induced cancerous EMT in SKOV3 and 3AO cell lines; in turn, this repression upregulated the expression of Id-1 at both the mRNA and protein level, thus expanding our knowledge of the role of miR-29b in the response to TGFβ1. To the best of our knowledge, this is the first study exploring the role of miR-29b in TGFβ1-induced cancerous EMT in human ovarian cancer cells. Our limited data demonstrate that miR-29b is downregulated in TGFβ1-induced cancerous EMT and identify Id-1 as a novel player in the TGFβ1/miR29b pathway. Further studies will be required to determine the specific significance of these molecules in ovarian cancer progression and metastasis.

TGFβ1 is capable of inducing EMT in a variety of human cancer cells, including ovarian cancer cells [59]. Given the crucial role of TGFβ1 in mediating cancerous EMT, extensive research is focusing on the specific mechanism by which TGFβ1 triggers the reversible but decisive transition in cancer metastasis. The effects of TGFβ signaling are transduced via binding to transmembrane serine-threonine type I (TbRI) and type II (TbRII) receptors. TbRII transphosphorylates TbRI, and the latter activates receptor-regulated Smad proteins; these Smad proteins then conduct signals from the TGFβ receptors to the nucleus, where they interact with various transcription factors and transcriptional co-activators or co-repressors, thus regulating target gene transcription. Recent studies have identified two main classes of signaling pathways that are responsible for the mediation of EMT by TGFβ1: the canonical Smad signaling pathway and various noncanonical Smad-independent pathways, including the ERK1/2, PI3K, JNK, and P38 MAPK pathways. Intriguingly, recent studies also suggest a potential connection between the canonical Smad signaling pathway and miR-29b. For instance, Qin and colleagues demonstrated that Smad3 interacted with the miR-29b promoter using a chromatin immunoprecipitation (ChIP) assay and further found that the interaction was markedly increased after TGFβ1 treatment, indicating that miR-29b may be a transcriptional target of TGF-β/Smad3 signaling [60]. Several other studies have demonstrated that miR-29 is repressed or absent in a number of diseases associated with fibrosis in the heart, lung, and liver, and that the levels of miR-29 can be reduced by TGFβ1 treatment [61-63]. Collectively, these studies demonstrate that miR-29b can serve as a TGFβ1/Smad-associated micro-RNA. However, it should also be noted that all of the abovementioned studies were performed using TGFβ1-induced fibrosis models. To our knowledge, miR-29b and the Smad signaling pathway have not yet been shown to interact in a TGFβ1-induced cancerous EMT model. Therefore, whether the miR-29b repression observed in our TGFβ1-induced EMT model of ovarian cancer is correlated with the Smad signaling pathway or with other non-Smad signaling pathways remains undetermined. In addition, the signaling mechanism by which TGFβ1 regulates miR-29b expression is also awaiting full elucidation. Undoubtedly, further studies targeting these specific questions will lead to a better understanding of the role of miR-29b as well as the biology of cancerous invasion and metastasis.

Taken together, our findings demonstrate for the first time that Id-1 can facilitate the EMT process induced by TGFβ1 in human ovarian cancer cells associated with the repression of miR-29b, which is responsible for the upregulation of Id-1 at both the mRNA and protein levels. Moreover, Id-1 levels are also correlated with pathological mesenchymal behaviors,

such as increased migration and invasion in human ovarian cancer cells. Further studies targeting Id-1 and the mechanism of miR-29b regulation by TGFβ1 induction will provide promising and feasible options for the treatment and prevention of human ovarian cancer.

Conflicts of Interest

The authors declare that no potential conflicts of interest exist.

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