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Semaphorin 3A inhibits tumor progression via the downregulation of Lin28B in ovarian cancer

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Semaphorin 3A (Sema3A) has recently been proven to play an essential role in tumorigenesis. Here, the role of Sema3A in ovarian cancer is explored. The prognostic value of Sema3A was evaluated using the Kaplan-Meier plotter database, and stable expression cells were established by the delivery of lentivirus harboring SEMA3A cDNA or shRNA into OVCA433 and SKOV3 cells, respectively. Then CCK-8 assay, colony-formation assay, wound-healing assay, and Transwell assay were utilized to verify the effect of Sema3A on tumorigenesis. Co-cultures of ovarian cancer cells (OVCA433 and SKOV3) with a conditional medium collected from the established cells were further utilized to confirm the function of Sema3A. Then, the RNA-seq assay was adopted to explore the underlying mechanism. The results demonstrated that low expression of Sema3A was predictive of poor overall survival in patients with ovarian cancer. Functional experiments revealed that Sema3A inhibited proliferation, migration, and invasion in ovarian cancer cells. Secreted Sema3A in a conditioned culture medium also exhibited an anti-tumor effect in ovarian cancer cells. RNA-seq assay suggested that focal adhesion and Lin28B were involved in regulating Sema3A. Rescue assays further verified that Lin28B/ROCK1 axis was vital in the regulation of Sema3A and Lin28B significantly upregulated ROCK1 through let-7g microRNA. The presented data indicate that Sema3A inhibits proliferation and metastasis via the downregulation of Lin28B/ROCK1 in ovarian cancer.

Key words: Sema3A; Lin28B; focal adhesion; ROCK1; tumorigenesis; ovarian cancer

Ovarian cancer is the fifth leading cause related to cancer among women, which has the worst prognosis and the highest mortality among gynecological cancers [1]. Epithelial ovarian cancer (EOC) is the most universal type accounting for 90% of ovarian cancer, in which the most prevalent is serous ovarian carcinoma [2]. Most patients are diagnosed at advanced stages (stage III or IV) due to asymptomatic growth and a lack of effective diagnostic methods. Although great progress has been made, the prognosis for patients with advanced EOC remains poor. Therefore, understanding the molecular mechanisms that lead to EOC is extremely urgent for developing new diagnostic techniques and improving the clinical treatment to facilitate the prognosis of patients with ovarian cancer.

Semaphorins are a large class of secretory and membrane-bound proteins, originally distinguished as axon guide factors [3]. In recent years, people have realized that semaphorins are closely related to angiogenesis, cell migration, organogenesis, and immune response [4, 5]. Semaphorin 3A (Sema3A) is a member of the family and serves as a secretory protein. Recent studies have indicated that Sema3A affects the biological behavior of malignancies. For instance, Sema3A plays an inhibitory role in head and neck squamous cell carcinoma, non-small-cell lung cancer (NSCLC), and oral cancer [6–8]. Moreover, Sema3A may be used as a novel type of therapeutic agent [9]. However, Sema3A is highly expressed and positively correlated with cell migration, metastasis, and poor prognosis in pancreatic cancer, hepatocellular cancer, and glioblastoma [9–11]. It has been reported that decreased expression of Sema3A is related to poor prognosis of EOC [12], suggesting that Sema3A may be a tumor suppressor in ovarian cancer, but the specific function and mechanism of Sema3A in EOC have not been clarified.

Lin28 family is a highly conserved RNA-binding protein, including two homologous members, Lin28A and Lin28B. Lin28 blocks the expression of let-7 and forms a dual-negative feedback loop, which is involved in multiple biological functions, such as tumorigenesis, immune destruction,

cell proliferation, cancer stem cell biology, and chemotherapy resistance [13–15]. A large number of studies have shown that high expression of Lin28B in human tumors is associated with poor clinical prognosis [16–18]. Recent studies have demonstrated that overexpression of Lin28B increases malignant capability and inhibits apoptosis in ovarian cancer. High expression of Lin28B is also associated with chemoresistance and adverse outcomes in ovarian cancer [19–22]. All these results indicate that Lin28B exerts a promoting role in ovarian cancer occurrence and development. HMGA2 is a downstream target of Lin28B. Lin28B enhances HMGA2 expression via inhibition of let-7 and the Lin28B-let-7-HMGA2 axis is involved in multiple tumorigeneses [23–25].

Focal adhesion is a necessary step in tumor metastasis. Rho kinases (ROCK1 and ROCK2) are involved in the assembly and turnover of the adhesion complex, thus regulating cell motility and proliferation [26]. Numerous studies have reported that ROCK1 serves as an essential factor in the metastasis of various cancer [27–29]. ROCK1 is also upregulated in ovarian cancer, which promotes tumor progression and is considered a new prognostic marker [30, 31].

In this study, we demonstrated that Sema3A significantly inhibited proliferation, migration, and invasion in ovarian cancer cells. Mechanism studies showed that the inhibitory effect of Sema3A on tumors was mediated by the Lin28B signaling pathway. These findings illustrated the molecular basis and biological function of Sema3A in ovarian cancer and may provide new strategies for EOC treatment.

Materials and methods

Cell culture. Human ovarian cancer cell lines SKOV3 and OVCA433, human embryonic kidney 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The ovarian cancer cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA). 293T cells were cultured in DMEM medium (Sigma, St. Louis, MO, USA) with 10% FBS. All cells were maintained in a 37 °C cell culture incubator with 5% CO_2.

Plasmid construction, cell transfection, and viral infection. The plasmid used to enhance Sema3A expression was constructed by inserting *SEMA3A* cDNA into the pCDH-CMV-MCS-EF1-puro vector. Lipofectamine 2000 was used for virus packaging according to the manufacturer's instructions. The ovarian cancer cell line OVCA433 was used for the stable introduction of *SEMA3A* cDNA. The infected cells were selected with puromycin (1.5–2.0 μg/ml) for 14 days, and the established cell line was named OVCA433 Sema3A. The control cells were infected with an empty vector, named OVCA433 Vector.

Two DNA oligonucleotides were designed to generate small hairpin RNA (shRNAs) against the open reading frame of *SEMA3A* mRNA and inserted into pLKO.1 vector to inhibit Sema3A expression. The target sequences were 5'-GCACAGAAGTCTGGTGAATAA-3' (shSema3A#1) and 5'-GCAATGGAGCTTTCCACTAAG-3' (shSema3A#2). The control plasmid was similarly constructed by directly inserting shRNA targeting firefly luciferase (5'-CTTACGCTGAGTACTTCGA-3') into pLKO.1 (shLuc). The ovarian cancer cell line SKOV3 was used for the stable introduction of *SEMA3A* shRNA. The infected cells were selected with puromycin (1.5–2.0 μg/ml) for 14 days, and the established cell lines were named SKOV3 shLuc, SKOV3 shSema3A #1, and SKOV3 shSema3A #2.

The plasmid containing *LIN28B* cDNA and *GFP* gene was purchased from HanBio Technology Co. Ltd. (Shanghai, China). A flag tag was inserted into the 3' terminus of *LIN28B* cDNA. Virus production and infection were performed according to the above protocol and the GFP-positive clones were selected under a fluorescence microscope.

RNA isolation and qRT-PCR. Total RNA was isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then 1 μg of RNA was reverse transcribed into cDNA using PrimeScript RT Reagent Kit (Takara, Otsu, Japan). qRT-PCR was subsequently performed using the FastStart Universal SYBR Green Master (ROX) kit (Roche, Indianapolis, IN, USA). With *GAPDH* as the internal control, PCR conditions were 95 °C for 15 s, 60 °C for 1 min, and then followed by 40 cycles of 95 °C for 5 s, 60 °C for 1 min, and 95 °C for 5 s, and 60 °C for 10 s. The primer sequences used were as follows: *SEMA3A*: forward, 5'-GTGCCAAGGCTGAAATTATCCT-3' and reverse, 5'-CCCACTTGCATTCATCTCCTTCT-3'; *GAPDH*: forward, 5'-TCCTCTGACTTCAACAGCGACAC-3', and reverse, 5'-CACCCTGTTGCTGTAGCCAAATTC-3'. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. All the experiments were repeated three times.

Western blotting. Whole cells were collected and lysed using RIPA lysis buffer (Beyotime Biotechnology, Jiangsu, China). Protein concentration was determined by the BCA protein assay kit (Beyotime Biotechnology, Jiangsu, China). Then the samples were adjusted to the same concentration with loading buffer, separated by SDS-PAGE, and transferred to PVDF membrane (Millipore, Billerica, MA, USA). After blocking, the membrane was incubated with diluted primary antibodies at 4 °C overnight and then incubated with appropriate secondary antibody at room temperature for 1 h. β-actin was used as a loading control. Antibody binding was detected using the enhanced chemiluminescence (ECL) detection kit (Millipore, Billerica, MA, USA). Primary antibodies utilized are listed below: Sema3A (ab23393, Abcam), Lin28B (4196, CST), ROCK1 (ab134181, Abcam), HMGA2 (A2972, ABclonal), Slug (9585, CST), and β-actin (A5316, Sigma-Aldrich).

Cell proliferation assay. Cells were inoculated into 96-well plates (1×10^3 cells/well), and 5 replicates were set in each group. Cell counting kit CCK-8 (Dojindo, Osaka, Japan) was used to measure cell viability. At the time point of 0, 24, 48, 72, and 96 h after seeding, 10% CCK-8 solution

was added to each well and incubated for 2 h. Cell viabilities were determined using a microplate reader to measure the absorbance at 450 nm. All the experiments were repeated three times.

Colony formation assay. Cells were inoculated in the 6-well plates (200 cells/well). The cells were further cultured in the incubator for two weeks, and the culture medium was changed every 5 days. Then the formed clones were stained with 0.1% crystal violet in methanol. After taking pictures, the clones were counted and analyzed statistically.

Wound healing assay. Cells were seeded in the 12-well plates. When the cells were approximately 100% confluent and aggregated to form a monolayer, a scratch was created using a 200 μl pipette tip, and cell debris was removed by washing with 1× PBS three times. Then the fresh medium without serum was added for cell culture. At the indicated time points, the scratches were photographed with a microscope. The percentage of wound closure was evaluated using Image-Pro Plus V6.2.

Cell migration and invasion assay. For invasion analysis, 60 μl diluted Matrigel (Corning, NY, USA) was placed on the upper chamber of a 24-well Transwell plate before seeding cells. Then 5×10^4 cells were suspended in 200 μl serum-free culture medium and inoculated in the upper chamber, and a complete culture medium with 10% fetal bovine serum was added to the lower chamber. After 48 h, the invaded cells at the lower surface of the chamber were fixed and stained with 0.1% crystal violet in methanol, 5 random microscope fields (magnification ×200) were photographed, and counted.

In the cell migration assay, 2×10^4 cells in 200 μl serum-free culture medium were seeded in the upper chamber without Matrigel precoating. The following steps were performed as in the invasion assay.

Conditioned medium (CM) collection and treatment. Cells with Sema3A overexpression or knockdown were plated in a 10 cm dish and cultured overnight. The culture medium was replaced by a fresh medium without FBS on the next day and cells were cultured for another 48 h. Then the medium was collected and centrifugated to remove cell debris.

OVCA433 cells were cultured with CM from Sema3A overexpression cells and SKOV3 cells were cultured with CM from Sema3A knockdown cells. CCK-8 assay was utilized to examine cell proliferation and Transwell assay was utilized to examine the metastatic ability.

RNA-seq and bioinformatic analysis. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from OVCA433 Sema3A cells and Vector cells, and the concentration, quality, and integrity of RNA were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA) was used to purify mRNA, synthesize cDNA, remove enzymes and hybridize, and finally generate sequencing files. After purification, the library fragments were subjected to highly sensitive DNA analysis on the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). The sequencing library was sequenced on the HiSeq platform (Illumina, San Diego, CA, USA), and bioinformatic analysis was performed by Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

Co-IP. SKOV3 cells with stable Lin28B expression were seeded in 15 cm dishes for 48 h; then, the cells were harvested and lysed using a lysis buffer containing a proteinase inhibitor cocktail. The supernatants were collected by centrifugation and incubated with an anti-Flag antibody (F1804, Sigma, USA) for 1 h at 4 °C. Then protein G agarose beads (Roche, USA) were added and incubated overnight at 4 °C. After sufficient washing, the bound proteins were eluted in 2× loading buffer and analyzed by western blot.

miRNA Transfection. Hsa-let-7g mimics and negative control were purchased from RiboBio (Guangzhou, China). Cells were seeded in a 6-well plate and incubated overnight. The transfection was performed using a LipoFilter according to the manufacturer's instructions. After transfection for 48 h, cells were harvested for western blot assay.

Statistical analysis. All the data were expressed as mean ± standard deviation (SD), t-test was applied to compare the difference data between the two groups, and the data between the three groups were analyzed and compared by one-way ANOVA. All statistical analyses were conducted in GraphPad Prism 7.0. A p-value <0.05 is referred to as statistically significant.

Results

The prognostic value of Sema3A in ovarian cancer and establishment of stable expression cell lines with *SEMA3A* cDNA and shRNA. A previous study on a total of 125 patients with ovarian cancer has shown that low Sema3A level was associated with poor prognosis [12]. In the present study, we further estimated the prognostic value of Sema3A by analyzing the Kaplan-Meier plotter database and found that patients expressing higher Sema3A showed significantly longer overall survival in ovarian cancer (Figure 1A), suggesting the importance of Sema3A in ovarian cancer. To further investigate the biological function of Sema3A in ovarian cancer, we established stable expression cell lines by introducing *SEMA3A* cDNA into OVCA433 cells to enhance Sema3A expression and introducing two shRNAs against *SEMA3A* (shSema3A #1 and shSema3A #2) to SKOV3 cells to attenuate Sema3A expression. Then the expression of Sema3A was determined by qRT-PCR and western blot respectively. As shown in the results, Sema3A was significantly upregulated in OVCA433 Sema3A cells (Figures 1B, 1C) and significantly downregulated in the knockdown cells (Figures 1D, 1E).

Sema3A inhibits proliferation in ovarian cancer cells *in vitro*. To determine the effect of Sema3A on cell proliferation, CCK-8 assay and colony formation assay were conducted. The results showed that the upregulation of Sema3A significantly

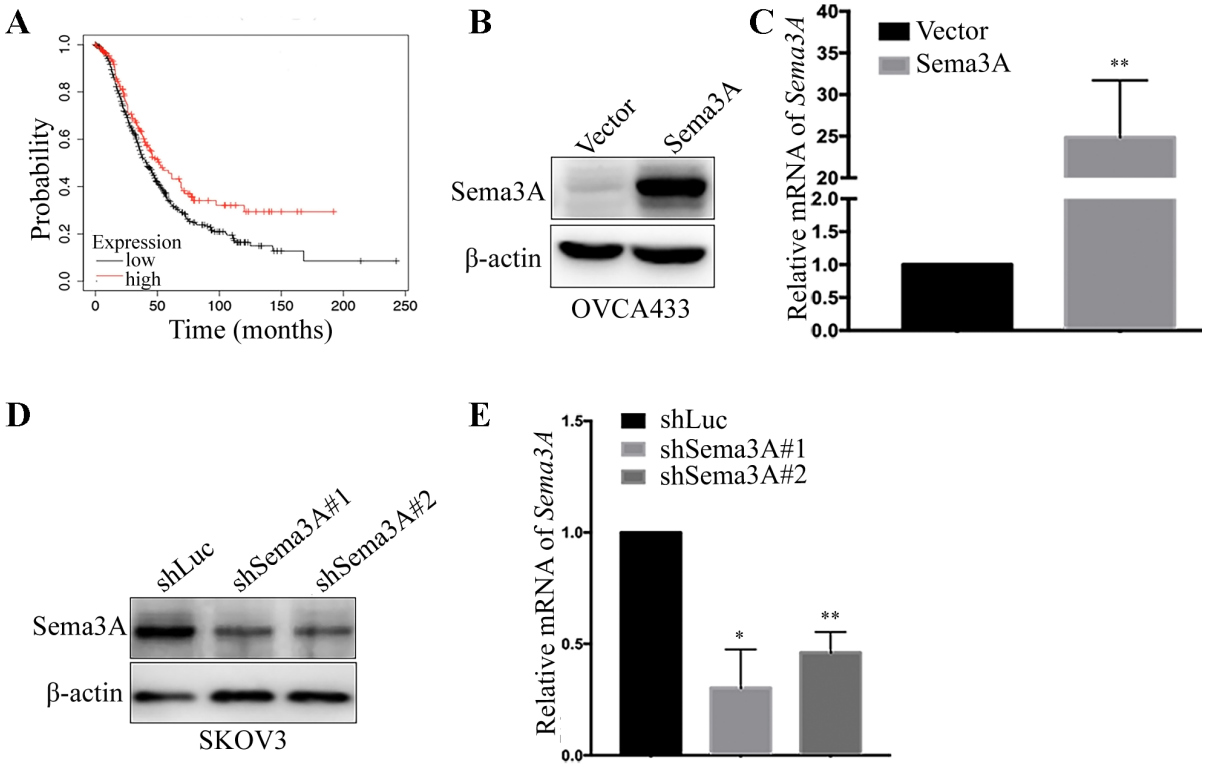

Figure 1. Sema3A predicts favorable outcomes in patients with ovarian cancer and the establishment of Sema3A overexpression and knockdown cells. Patients with higher Sema3A showed longer overall survival than those with lower Sema3A levels (HR=0.73 (0.57–0.94), p=0.013). A) Sema3A was significantly upregulated in OVCA433 cells infected with *SEMA3A* cDNA at the protein level. B) The efficiency of Sema3A overexpression was analyzed by qRT-PCR (p<0.01). C) Sema3A was significantly decreased in SKOV3 cells harboring *SEMA3A* shRNAs at the protein level. D) The efficiency of Sema3A knockdown was analyzed by qRT-PCR (*p<0.05, **p<0.01).**

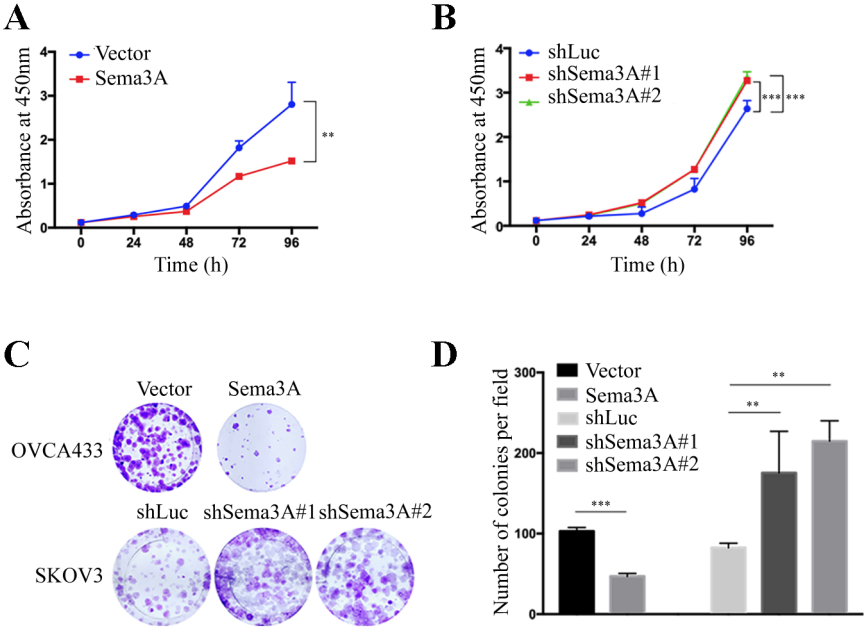

Figure 2. Sema3A inhibits proliferation in ovarian cancer cells. A) Overexpression of Sema3A inhibited proliferation in ovarian cancer cells (p<0.01). B) Knockdown of Sema3A promoted proliferation in ovarian cancer cells (***p<0.001). C) Clone formation ability was decreased in OVCA433 Sema3A overexpression cells and increased in Sema3A knockdown cells. D) Statistical analysis of the number of clones. Each experiment was conducted in triplicate; bar graphs are represented as mean ± SD; two-tailed unpaired student's t-test, **p<0.01, ***p<0.001**

repressed cell growth (Figure 2A) and the knockdown of Sema3A promoted cell proliferation (Figure 2B). Moreover, the clones formed by OVCA433 Sema3A cells were fewer and smaller than those formed by OVCA433 Vector cells, while cells harboring *SEMA3A* shRNAs had increased colony formation ability (Figures 2C, 2D). These data indicated that Sema3A inhibited proliferation in ovarian cancer cells.

Sema3A significantly reduces metastasis in ovarian cancer cells. To assess the function of Sema3A on migration and invasion in ovarian cancer cells, wound healing assay and Transwell assay were performed. The result of the wound healing assay showed that overexpression of Sema3A obviously eliminated motility, and the wound closure rates of Sema3A knockdown cells were significantly higher than that of the control group (Figures 3A–3C). The result of the Transwell assay also indicated that the capacity of migration and invasion was reduced in the Sema3A overexpression cells and elevated in Sema3A silencing cells (Figures 3D–G). Taken together, these results suggested that Sema3A reduced the metastatic ability in ovarian cancer cells.

Effect of Sema3A secreted from conditional medium (CM) on proliferation and invasion capacity of ovarian cancer cells. To investigate the effect of secreted Sema3A on OVCA433 and SKOV3 cells, we collected CM from Sema3A overexpression and knockdown cells. Compared to CM from OVCA433 Vector cells, CM from OVCA433 Sema3A cells inhibited the growth of OVCA433 cells (Figure 4A). Consistent with this, we found that co-culture of OVCA433 cells with CM from OVCA433 Sema3A weakened the invasion capacity compared to co-culture with OVCA433 Vector cells in a Matrigel-coated Transwell assay (Figures 4C, 4D). As displayed in Figure 4B, the proliferation capacity of SKOV3 cells co-cultured with CM from Sema3A knockdown cells had a significant enhancement compared to that with CM from Sema3A shLuc cells. We then observed that CM from shSema3A cells significantly promoted invasion in SKOV3

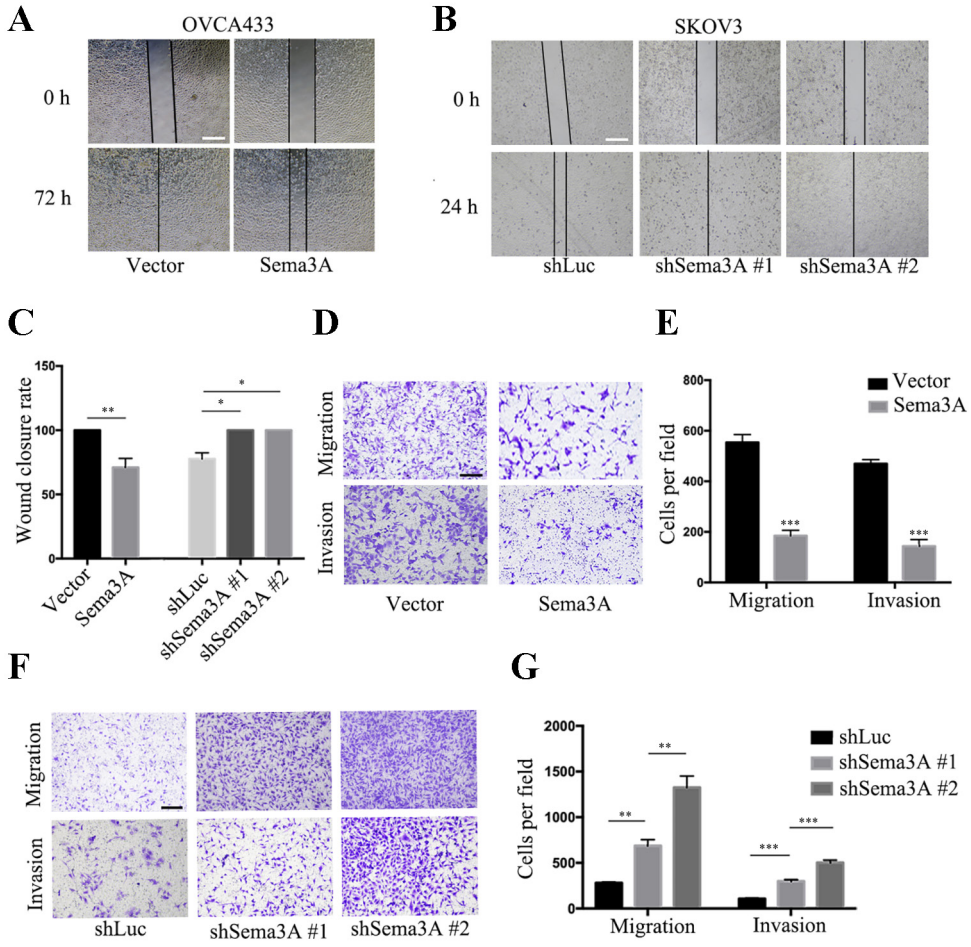

Figure 3. Sema3A inhibits metastasis in ovarian cancer cells. A) Sema3A overexpression reduced cell motility in OVCA433 cells in wound healing assay (Scale bar: 500 μm). B) Sema3A knockdown promoted cell motility in SKOV3 cells (Scale bar: 500 μm). C) Statistical analysis of wound closure rate (*p<0.05, **p<0.01). D, E) The migration and invasion ability were decreased in Sema3A overexpression cells. (Scale bar: 200 μm, ***p<0.001). F, G) Cell migration and invasion capacity were increased upon Sema3A knockdown (Scale bar: 200 μm, **p<0.01; ***p<0.001).

cells (Figures 4D, 4E). These results depicted that secreted Sema3A could suppress the proliferation and invasion of ovarian cancer cells in a paracrine manner.

Focal adhesion and Lin28B are regulated by Sema3A. To identify the potential genes and signaling pathways regulated by Sema3A, we conducted transcriptomic sequencing analysis (RNA-seq) in OVCA433 Sema3A cells and the corresponding control cells. Sequencing results showed that a total of 328 different genes were detected, including 55 upregulated genes and 273 downregulated genes (Figure 5A). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment results showed that focal adhesion was involved in Sema3A's regulation (Figure 5B). Numerous studies have revealed that Sema3A could regulate focal adhesion to affect cell motility in different manners [32–34]. Our RNA-seq data identified that ROCK1, a major component involved in focal adhesion assembly, was downregulated in Sema3A overexpression cells. And further validation assay using qRT-PCR and western blot also indicated that ROCK1 was decreased in Sema3A overexpression cells (Figures 5C, 5D). Several studies have shown that focal adhesion could be influenced by the expression of Lin28B [25, 35, 36]. Moreover, Lin28B expression was significantly altered in RNA-seq data. Then we verified the expression of Lin28B at mRNA and protein levels. As shown in Figure 5C, *LIN28B* mRNA was downregulated in Sema3A overexpression cells, which was consistent with the RNA-seq results. And Lin28B was also downregulated in Sema3A overexpression cells at the protein level (Figure 5D). Furthermore, Lin28B and ROCK1

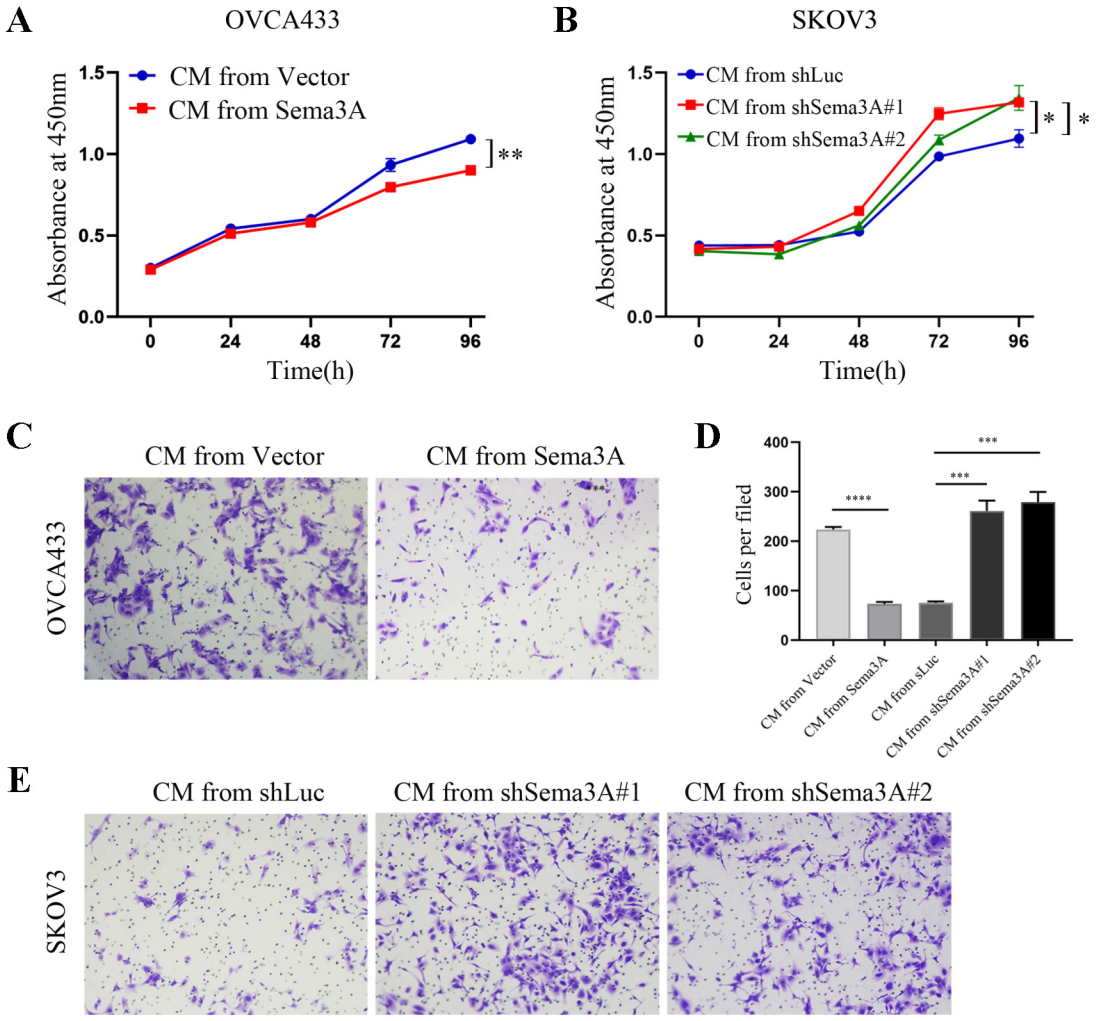

Figure 4. Secreted Sema3A in conditional medium inhibited cell proliferation and metastasis in ovarian cancers. A) The cell proliferation rate of OVCA433 cells co-cultured with CM from the OVCA433 Vector and Sema3A cells was evaluated by CCK-8 assay. B) The cell proliferation rate of SKOV3 cells co-cultured with CM from SKOV3 shLuc and shSema3A cells was evaluated by CCK-8 assay (*p<0.05). C, D) Matrigel-coated Transwell assay revealed the invasive capacities of OVCA433 cells co-cultured with CM from OVCA433 Vector and Sema3A cells (**p<0.0001). D, E) Matrigel-coated Transwell assay depicted the invasive capacities of SKOV3 cells co-cultured with CM from SKOV3 shLuc and shSema3A cells (***p<0.001).**

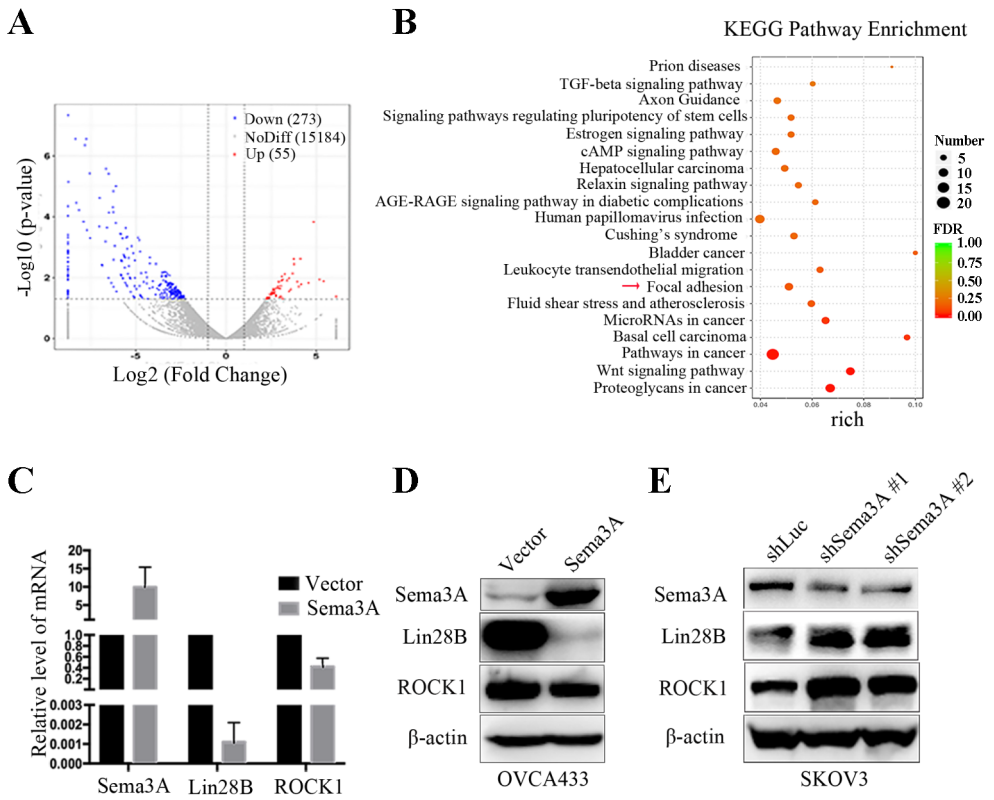

Figure 5. The focal adhesion pathway and Lin28B are enriched in RNA-seq analysis. A) Volcano plot of gene expression (Sema3A overexpression versus Vector; fold change, ≥ 2; p<0.05). B) KEGG pathway enrichment of differentially expressed genes. C) Validation of the selected genes using qRT-PCR. D) Validation of the selected genes at the protein level in Sema3A overexpression cells. E) Measurement of the selected genes in Sema3A knockdown cells.

were upregulated in Sema3A knockdown cells (Figure 5E). The above results indicated that ROCK1 and Lin28B acted as downstream targets of Sema3A.

Ectopic expression of Lin28B abrogates the inhibitory effect of Sema3A in ovarian cancer cells. To further explore the underlying mechanism regulated by Sema3A in ovarian cancer, *LIN28B* cDNA was delivered into Sema3A overexpression cells. The results showed that expression of Lin28B was markedly enhanced in protein level (Figure 6A). Moreover, the repressive effect of Sema3A on cell growth was abrogated by Lin28B induction (Figure 6B), and the reduced metastatic ability in Sema3A overexpression cells was partially restored by Lin28B overexpression (Figures 6C, 6D). Mechanism analysis showed that decreased expression of ROCK1 in Sema3A overexpression cells was recovered by Lin28B induction (Figure 6E). As a major target of Lin28B, HMGA2 was also downregulated in Sema3A overexpression cells, and elevated by Lin28B overexpression. Since HMGA2 could directly bind to the *SLUG* promoter and triggered EMT [37], we then detected the expression of Slug. The results indicated that the expression of Slug was reduced in Sema3A overexpression cells, but recovered by forced expression of Lin28B. The above results indicated that Lin28B was sufficient to reverse the inhibitory effect of Sema3A on ovarian cancer, suggesting that downregulation of Lin28B was required for Sema3A's function.

Suppressing the expression of Lin28B restored the effects of Sema3A knockdown in ovarian cancer cells. To confirm the relationship between Sema3A and Lin28B in ovarian cancer, *LIN28B* shRNA was transfected into Sema3A knockdown SKOV3 cells. Western blot data demonstrated that the expression of Lin28B was markedly increased in Sema3A silencing cells, transfection of shLin28B abolished the expression of Lin28B (Figure 7A). Suppression of Lin28B repressed proliferation and metastasis induced by Sema3A knockdown cells (Figures 7B–7D). Expression of Mechanism research revealed that Sema3A-depleted cell lines exhibited significant upregulation of ROCK1, HMGA2, and Slug, and the upregulation was inhibited by shLin28B (Figure 7E). This finding further suggested that Lin28B/ROCK1 axis was involved in Sema3A-regulated tumor progression in ovarian cancer.

Lin28B regulates the expression of ROCK1 via let-7g. A previous study revealed that Lin28A, a homolog of Lin28B could interact with ROCK2 by direct binding to promote the

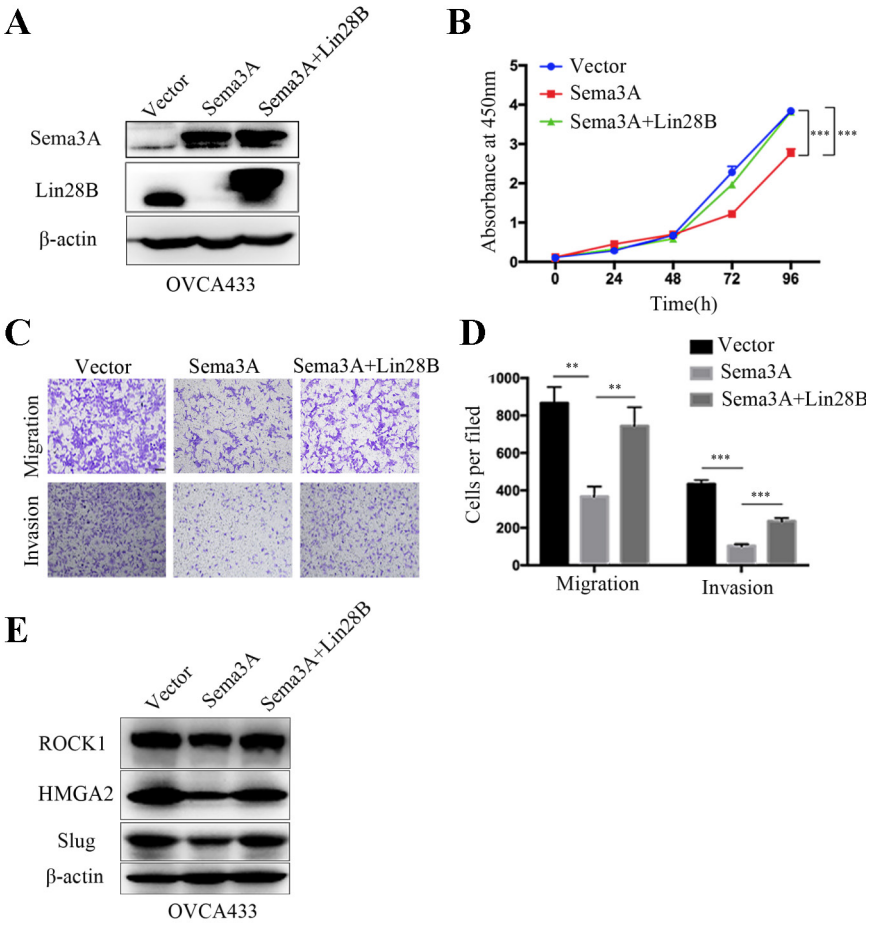

Figure 6. Lin28B is required for Sema3A's function. A) Ectopic expression of Lin28B in Sema3A overexpression cells and validation of Lin28B expression using western blot. B) Lin28B rescued Sema3A-inhibited cell growth (*p<0.001). C, D) Lin28B partially restored Sema3A-inhibited metastasis (Scale bar: 200 μm, **p<0.01, ***p<0.001). E) Lin28B enhanced the expression of ROCK1, HMGA2, and Slug, which were decreased by Sema3A.**

malignancy of ovarian cancer (the interaction of Lin28A/Rho-associated coiled-coil containing protein kinase2 accelerates the malignancy of ovarian cancer). To confirm whether Lin28B could regulate the expression of ROCK1 in the same manner, the co-IP assay was performed in SKOV3 Lin28B overexpression cells using an anti-Flag antibody. The results demonstrated that Lin28B does not directly interact with ROCK1 in ovarian cancer (Figure 8A). Let-7 family microRNAs are vital in the Lin28B-regulated signaling pathway. To further identify the mechanism through which Lin28B regulated ROCK1, hsa-let-7g mimics were transfected into Lin28B overexpression cells, the results demonstrated that the introduction of hsa-let-7g mimics abrogated the upregulation of ROCK1 induced by Lin28B, and the inhibitory effect was concentration dependent. HMGA2 was utilized as a positive control (Figure 8B). Those results demonstrated that ROCK1 was regulated by Lin28B via let-7 microRNA.

Discussion

A previous study demonstrated that Sema3A was downregulated in tumor tissues and decreased expression of Sema3A predicted adverse prognosis in ovarian cancer [12]. KM plotter data also depicted that low expression of Sema3A predicted poor overall survival (Figure 1A), suggesting that Sema3A was a potential tumor suppressor in ovarian cancer. In this study, we examined the specific function of Sema3A in ovarian cancer and demonstrated that Sema3A indeed inhibited cell proliferation, migration, and invasion *in vitro*. Furthermore, we found that secreted Sema3A in CM collected also decreased the metastasis and proliferation of ovarian cancer cells, suggesting that Sema3A could exert an anti-tumor effect both in an autocrine and paracrine manner.

Our findings are consistent with those of other solid tumors, including head and neck squamous cell carcinoma [6], NSCLC [7], and oral cancer [8]. Various downstream

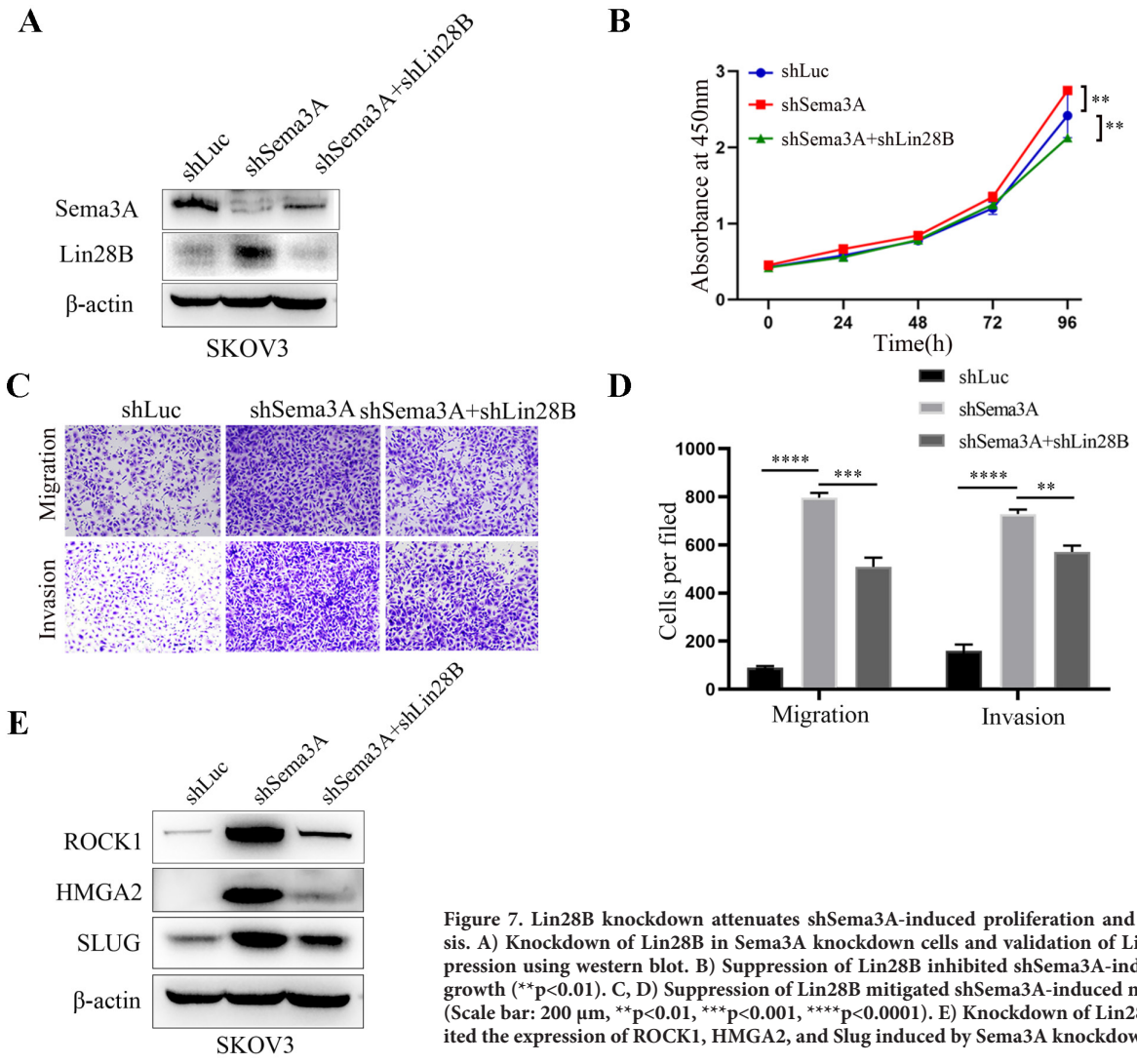

Figure 7. Lin28B knockdown attenuates shSema3A-induced proliferation and metastasis. A) Knockdown of Lin28B in Sema3A knockdown cells and validation of Lin28B expression using western blot. B) Suppression of Lin28B inhibited shSema3A-induced cell growth (p<0.01). C, D) Suppression of Lin28B mitigated shSema3A-induced metastasis (Scale bar: 200 μm, **p<0.01, ***p<0.001, ****p<0.0001). E) Knockdown of Lin28B inhibited the expression of ROCK1, HMGA2, and Slug induced by Sema3A knockdown.**

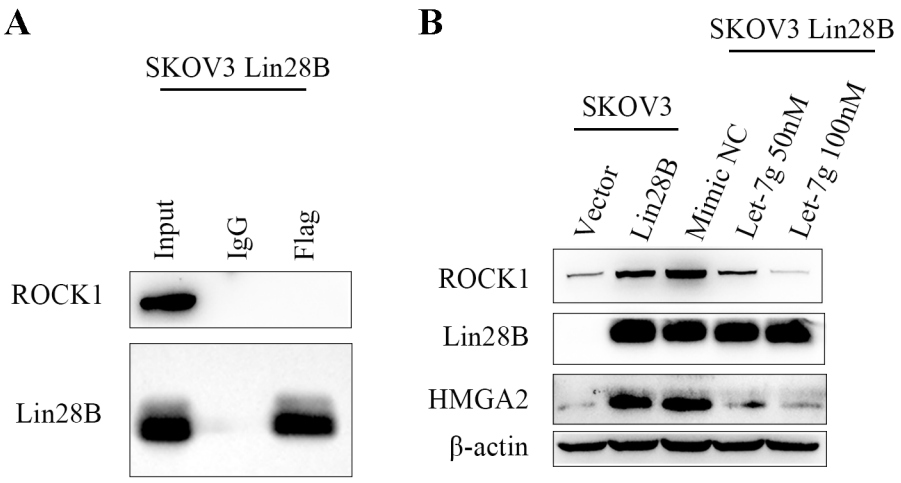

Figure 8. Lin28B regulates ROCK1 via let-7 microRNA. A) Lin28B could not directly interact with ROCK1 detected by the co-IP assay. B) ROCK1 was regulated by Lin28B via let-7 microRNA.

proteins, such as CapG, galectin-3, enolase 2, NF-kappa B, and SNAI2, are regulated by Sema3A in related tumors, affecting proliferation, migration, and apoptosis [6, 11]. However, the underlying mechanism of ovarian cancer is still unknown. Our RNA-seq results and the subsequent validation assay demonstrated that Lin28B was the downstream target of Sema3A. Lin28B is an ovarian cancer biomarker that contributes to tumor proliferation and migration and predicts poor prognosis [19, 38]. Many studies have demonstrated that Lin28B affects tumor development by altering the expression of downstream genes and negatively regulating the let-7 family [13, 15]. We further enhanced Lin28B expression in Sema3A overexpression cells and found that Lin28B rescued the inhibitory effect of Sema3A on cell proliferation and metastasis, suggesting that Lin28B was involved in Sema3A-regulated tumor progression. Functional annotation revealed that Sema3A targets were enriched in the focal adhesion pathway. ROCK1 is a crucial member of the focal adhesion assembly. Several studies have reported that ROCK1 exerts a vital role in the metastasis of various cancers, including NSCLC [27], cervical cancer [28], and prostate cancer [29]. Recent studies have indicated that the deletion of ROCK1 suppresses the signal transduction of focal adhesion kinase, resulting in the inability to form mature and stable adhesion plaques [39] and changing cell motility [26]. Inhibition of Rho-associated protein kinase (ROCK) can prevent Sema3A-mediated effects on cell migration and diffusion of adhesion plaques [32]. Our present study demonstrated that Sema3A inhibited ROCK1 and forced expression of Lin28B enhanced the expression of ROCK1 via let-7 microRNA. Moreover, the Lin28B target HMGA2 and subsequent Slug were reduced in Sema3A overexpression cells and restored by ectopic expression of Lin28B. However, the regulation mechanisms between Sema3A and Lin28B have still not been elucidated, requiring further research.

Our study uncovered that Sema3A acted as a tumor suppressor and inhibited cell proliferation and metastasis in ovarian cancer; Sema3A targets were enriched in the focal adhesion signaling pathway; Lin28B was downregulated by Sema3A; Lin28B-regulated ROCK1/HMGA2 axis via let-7 microRNA involved in Sema3A's function. In conclusion, Sema3A suppressed tumor progression via the downregulation of Lin28B, which may serve as a diagnostic biomarker and facilitate the clinical treatment for ovarian cancer patients.

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