Long Noncoding RNA LINC00858 Promotes the Progression of Ovarian Cancer via Regulating the miR-134-5p/TRIM44 Axis

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Primary research

Keywords: long non-coding RNAs (lncRNAs), cell proliferation, invasion, tumor growth

Posted Date: June 9th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-30879/v1

Abstract

Background

Ovarian cancer remains one of the most challenging areas of cancer research. Recent studies have shown that many long non-coding RNAs (lncRNAs) are abnormally expressed in ovarian cancer and involved in the pathological progress of ovarian cancer. In the present study, we aimed to investigate the role of lncRNA LINC00858 and the potential mechanism in ovarian cancer.

Methods

The qRT-PCR was used to measure the expression levels of LINC00858 and miR-134-5p in ovarian cancer tissue specimens and cell lines. Loss-of-function assays were performed to investigate the role of LINC00858 in ovarian cancer progression. MTT assay was carried out to measure cell proliferation. Transwell assays were performed to determine the migration and invasion of ovarian cancer cells. Biological information analysis and luciferase report gene assay were used to verify potential downstream genes of LINC00858. The xenograft mouse model was established to analyze tumor growth *in vivo*.

Results

Our results showed that LINC00858 was highly expressed in human ovarian cancer tissue specimens and cell lines. Loss-of-function assays showed that knockdown of LINC00858 significantly inhibited cell proliferation, migration and invasion of SKOV3 cells, and suppressed tumor growth in mouse xenograft models. Mechanistic studies revealed that LINC00858 acted as a sponge of miR-134-5p and then regulated the expression TRIM44 in SKOV3 cells. Furthermore, rescue experiments illustrated that inhibition of miR-134-5p restored the inhibitory effects of LINC00858 knockdown on ovarian cancer cell proliferation, migration and invasion. TRIM44 overexpression could counteract the inhibitory effects of miR-134-5p mimics on ovarian cancer cells.

Conclusion

In conclusion, these findings demonstrated that LINC00858 exerted oncogenic role in ovarian cancer, which was mediated by miR-134-5p/TRIM44 axis. Thus, LINC00858 might be a therapeutic target for the treatment of ovarian cancer.

1. Background

Ovarian cancer is the most fatal malignancy of female reproductive system [1]. Despite recent improvements in surgical resection and chemotherapy, the 5-year survival rate of patients with ovarian

cancer is still approximately 30–40%, which may be attributed to late diagnosis, chemoresistance and the metastasis [2]. Increasing evidence reveals that the tumorigenesis of ovarian cancer is a complicated process referring multiple epigenetic and genetic alterations [3, 4]. Consequently, better understanding of the molecular alterations and their functional significance may provide crucial insights on developing the approaches for diagnosis, treatment, and prevention of ovarian cancer.

Long non-coding RNAs (lncRNAs) are an abundant class of transcripts that do not encode proteins and are longer (> 200 bp) than other noncoding transcripts like microRNAs [5]. LncRNAs are thought to be modulators of expression patterns dictated by transcription regulators [6]. Some nuclear lncRNAs act in cis and regulate (activate or repress) genes expression in their immediate vicinity. LncRNAs localized to the cytoplasm interfere with translation, have catalytic activity, or act as miRNA sponges [7]. In recent years, more and more findings implicate that lncRNAs play crucial roles in cellular differentiation, cell lineage choice, organogenesis and tissue homeostasis [8]. Moreover, dysregulation of lncRNAs are observed in many pathological conditions such as cardiovascular diseases and cancers, and therefore provide novel biomarkers and pharmaceutical targets [9, 10, 11].

LncRNA LINC00858 is a kind of lncRNA with 2685 nucleotides length that locates in 10q23.1. It has been reported that LINC00858 is involved in tumorigenesis of several types of cancers through regulating tumor cell growth, proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) [12, 13, 14]. However, the roles and potential biological mechanisms of LINC00858 on ovarian cancer still remain to be expounded. In the current study, the role of LINC00858 in ovarian cancer and its relevant mechanisms were investigated both *in vitro* and *in vivo*.

2. Materials And Methods

2.1 Clinical tissues and cell culture

All methods used in the present study were conducted in accordance with the relevant guidelines and regulations approved by Huaihe Hospital of Henan University (Kaifeng, China). The collection and usage of clinical tissues were approved by the participants and they all signed the informed consents. In total, 46 paired tissue samples were obtained from patients who were diagnosed with ovarian cancer and confirmed by more than two senior physicians in the pathology department of Huaihe Hospital of Henan University. The patients underwent surgery between September 2017 and August 2018 without chemotherapy, radiotherapy, or hormonal therapy preoperatively.

Standard ovarian cancer cell lines including A2780, IGROV1, OVCAR3, and SKOV3 cells, and a normal human ovarian surface epithelial cell line HOSE 6.3 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultivated within RPMI-1640 that contains 10% FBS and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA). All cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

2.2 Cell transfection

Lentiviral vectors carrying small interfering RNA (siRNA) targeting LINC00858 (lv-LINC00858) and a negative control lentivirus (lv-NC) were prepared by Genechem (Shanghai, China). Then lv-LINC00858 or lv-NC was infected with SKOV3 cells according to the instruction. At 72 h post infection, the stable LINC00858-knockdown SKOV3 cells were selected.

The cDNA of TRIM44 was inserted into pcDNA3.1 vector to construct a TRIM44-overexpressing plasmid (pcDNA3.1-TRIM44), which was confirmed by gene sequencing. MiR-134-5p mimic, inhibitor, and the corresponding negative controls were obtained from GenePharma (Shanghai, China). According to the instructions, pcDNA3.1-TRIM44, pcDNA3.1, miR-134-5p mimic, control mimic, miR-134-5p inhibitor, and control inhibitor were transfected into the SKOV3 cells using Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA).

2.3 Cell proliferation assay

SKOV3 cells were seeded into 96-well flat bottomed plates at the density of 5×10^3 cells per well. After 0, 24, 48, 72 h cultivations, 20 μl MTT solution (5 mg/ml; Promega, Madison, WI, USA) was supplemented into each well and incubated for 4 h. The supernatants were then discarded, followed by the addition of 100 μl dimethylsulfoxide into each well. The absorbance value was measured at the wavelength of 490 nm using a microplate reader (Bio-Tek, Winooski, VT, USA).

2.4 Cell migration and invasion assays

SKOV3 cells in serum-free medium were seeded into the upper chamber of Boyden Chambers, while lower chambers were added with medium containing 10% FBS. After 24 h, cells remained on the inserts were cleared with a wet cotton swab. The cells moved to the lower side of the inserts were fixed with 10% methanol and then stained in crystal violet. Migration and invasion were quantified by taking mean and standard deviations of 4 non-biased image fields. The stained cells were observed under an inverted microscope, five fields were selected for the quantification of cell number.

2.5 qRT-PCR

Total RNA was isolated from tissues and cell lines using Trizol reagent following manufacturer's protocol (Invitrogen). After determining the RNA concentration using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), the RNA was used to generate cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). Then the qRT-PCR was performed using SYBR Green 2x Master Mix (Life Technologies) on ABI HT9600 from Applied Biosystems (Foster City, CA, USA) with the following reaction condition: predenaturation at 95°C for 30 s, and 40 cycles of denaturation at 95°C for 5 s, annealing at 57°C for 30 s. Finally, the expressions of target genes were measured using the $2^{-\Delta\Delta Ct}$ method relative to GADPH or U6.

2.6 Western blot

Total proteins were harvested from SKOV3 cells using RIPA buffer containing PMSF (protease and phosphatase inhibitors). Protein concentration was quantified using BCA protein assay kit. Proteins separated by 10% SDS-PAGE (Invitrogen) were electrically transferred onto PVDF membranes (Thermo Fisher Scientific) and then blocked in 5% defatted milk buffer. Membranes were incubated with primary antibodies against TRIM44 or β-actin (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After rinsing with TBST for 30 min, the membranes were incubated with Horse Radish Peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology) for 1 h at room temperature. Chemiluminesence western blotting reagent (Cell Signaling Technology) was used to visualize the immunoreactive proteins. Protein bands were quantified using Image J software.

2.7 Target prediction

LncRNATargets (http://www.herbbol.org:8001/lrt/) and Starbase (http://starbase.sysu.edu.cn/) were performed to identify specific miRNAs regulated by LINC00858. TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/) databases were used to predict putative target genes of miR-134-5p.

2.8 Luciferase reporter assay

The fragment of LINC00858 containing the predicted binding site with miR-134-5p and the corresponding mutant were respectively subcloned into the pmiRGLO Vector (Promega) to generate pmiRGLO-LINC00858-Wt and pmiRGLO-LINC00858-Mut plasmids. Meanwhile, the fragment from 3'UTR of TRIM44 containing the predicted miR-134-5p binding site and the corresponding mutant were inserted into pmiRGLO Vector to construct pmiRGLO-TRIM44-Wt and pmiRGLO-TRIM44-Mut plasmids. Afterwards, we co-transfected pmiRGLO-LINC00858-Wt/-Mut or pmiRGLO-TRIM44-Wt/-Mut with miR-134-5p mimics or control mimics into SKOV3 cells with Lipofectamine 2000. The luciferase activity assay was performed after 48 h post-transfection using a dual-luciferase reporter assay system (Promega).

2.9 *In vivo* xenograft model

Animal studies were approved by the Institutional Animal Care and Use Committee at Binzhou Medical University Hospital. Six-week-old male BALB/c Nude mice were purchased from Charles River Laboratories (Beijing, China). The lv-LINC00858 or lv-NC infected SKOV3 cells were subcutaneously injected in the right flank of the nude mice. Tumors were measured every 7 days, and tumor volume was calculated. Four weeks after inoculation, the mice were sacrificed and the tumors were collected for further experiments.

2.10 Statistical analysis

Statistical significance was analyzed by *t*-test and one-way ANOVA using GraphPad Prism Ver.5 (GraphPad Software, San Diego, CA, USA). *P* values less than 0.05 were considered statistically significant. Data were presented as mean ± SD of three independent experiments.

3. Results

3.1 LINC00858 is highly expressed in human ovarian cancer tissue specimens and cell lines

To examine if LINC00858 expression was altered in ovarian cancer, we firstly tested LINC00858 expression in human ovarian cancer tissues and their counterparts by qRT-PCR method. The Fig. 1A results showed an upregulation of LINC00858 in ovarian cancer tissues, compared to the adjacent non-tumor tissues. Similarly, increased LINC00858 expression was also detected in ovarian cancer cell lines (SKOV3, IGROV1, A2780 and OVCAR3 cells) when compared with that in the normal cell line (HOSE 6.3 cells). Among these ovarian cancer cell lines, LINC00858 was higher expressed in SKOV3 cells, therefore, we selected SKOV3 cells for the forthcoming experiments (Fig. 1B).

3.2 Down-regulation of LINC00858 inhibits the proliferation, migration and invasion of ovarian cancer cells

We then constructed stable LINC00858-knockdown cells through infection with lv-LINC00858. SKOV3 cells infected with lv-NC were used as a control. The qRT-PCR results showed that LINC00858 expression in lv-LINC00858-infected SKOV3 cells was approximately decreased by 0.79-fold compared with control group (Fig. 2A). And then, MTT assay was conducted to examine the role of LINC00858 in cell proliferation. The results revealed that knockdown of LINC00858 contributed to a reduction in the cell proliferation of SKOV3 cells than that of control cells (Fig. 2B). Cell migration assay showed that the migrative ability of SKOV3 cells in LINC00858 knockdown group was markedly reduced when compared with the control group (Fig. 2C). Similarly, cell invasion was significantly decreased after lv-LINC00858 infection in SKOV3 cells (Fig. 2D).

3.3 LINC00858 targeted miR-134-5p in ovarian cancer cells

Recent studies reported lncRNAs may function as miRNA sponges, thereby regulating the biological functions of miRNA. To choose miRNAs interacted with LINC00858, we analyzed the overlap from results of online software to predict potential miRNAs. We found that miR-134-5p was gotten as the candidate miRNA (Fig. 3A). As miR-134-5p has been demonstrated to possess tumor-suppressive effect, we next verified whether miR-134-5p interacted with LINC00858 using luciferase reporter assay. Co-transfection with miR-134-5p mimics and reporter vectors with the fragment of wide type LINC00858 markedly reduced luciferase activity in SKOV3 cells (Fig. 3B). Relative lower expression of miR-134-5p was observed in ovarian cancer cell lines compared with HOSE 6.3 cells (Fig. 3C). Furthermore, up-regulation of miR-134-5p was detected in lv-LINC00858 infected SKOV3 cells (Fig. 3D). In addition, MTT and transwell assays revealed that inhibition of miR-134-5p restored the inhibitory effects of LINC00858 knockdown on proliferation, migration and invasion of SKOV3 cells (Fig. 3E-3G).

3.4 miR-134-5p targeted TRIM44 in ovarian cancer cells

Next, we predicted the target genes of miR-134-5p using TargetScan (Fig. 4A). Among the predicted targets of miR-134-5p, TRIM44 was a cancer-related gene that has been demonstrated to be involved in

the cancer progression of ovarian cancer [15]. Thus, we selected TRIM44 for the further investigation of the interaction between TRIM44 and miR-134-5p. Luciferase reporter assay showed that co-transfection with miR-134-5p mimics and reporter plasmid containing wide type 3'-UTR of TRIM44 caused significant decrease in luciferase activity of SKOV3 cells (Fig. 4B). Ectopic expression of miR-134-5p significantly suppressed the mRNA and protein expression levels of TRIM44 in SKOV3 cells (Fig. 4C and 4D). Our results also showed that the protein and mRNA expression levels of TRIM44 were significantly up-regulated in ovarian cancer cell lines (Fig. 4E and 4F). Moreover, ectopic expression of TRIM44 restored the decreased proliferation, migration and invasion induced by miR-134-5p mimics in SKOV3 cells (Fig. 4G-4I).

3.5 Down-regulation of LINC00858 inhibits *in vivo* xenograft tumor growth

After subcutaneously injection with lv-LINC00858 or lv-NC infected SKOV3 cells, the tumor volume was detected. In the LINC00858 knockdown group, reduced tumor volume was observed on 3 and 4 weeks post infection (Fig. 5A). After dissection, a significant lower tumor weight was found in LINC00858 knockdown group, as compared to the control group (Fig. 5B). In addition, miR-134-5p expression was up-regulated, while TRIM44 expression was down-regulated in LINC00858 knockdown group compared with control group (Fig. 5C and 5D).

4. Discussion

The biological function and the underlying mechanism of LINC00858 in ovarian cancer have not been reported. In the current study, we first showed that down-regulation of LINC00858 inhibits the proliferation, migration and invasion of ovarian cancer cells, as well as attenuates tumor growth *in vivo*, further indicating the oncogene role of LINC00858 in ovarian cancer.

Recent studies have shown that a variety of lncRNAs are abnormally expressed in ovarian cancer and contribute to the pathological progress of ovarian cancer [16, 17]. LncRNA MALAT1 is upregulated in ovarian cancer tissues and indicates lower 5-year overall survival rate. MALAT1 inhibition impedes cell proliferation, invasion and metastasis, and promotes cell apoptosis of ovarian cancer cells [18]. LncRNA TINCR expression is higher in epithelial ovarian cancer tissues, silencing TINCR inhibits the malignant phenotype of epithelial ovarian cancer [19]. LncRNA PXN-AS1 expression is downregulated in pancreatic cancer samples and suppresses pancreatic cancer progression by acting as a competing endogenous RNA [20]. These lncRNAs act as either oncogenes or tumor suppressors, and serve as potential therapeutic targets.

LINC00858 is a novel lncRNA that has been demonstrated to be implicated in several cancers. LINC00858 expression is significantly up-regulated in colorectal cancer (CRC) tissues and associated with advanced clinical progression and poor prognosis. Knockdown of LINC00858 suppresses CRC cells proliferation, migration and invasion, and promotes apoptosis [13]. LINC00858 expression was found to be significantly upregulated in osteosarcoma tissues. Mechanism assays present that LINC00858 silencing significantly represses osteosarcoma cells proliferation and invasion *in vitro*, and inhibits tumor growth *in*

vivo [14]. In addition, overexpression of LINC00858 significantly promotes cell proliferation, invasion and the EMT process in lung cancer cells [12]. In accordance with previous studies, herein, we observed that LINC00858 was highly expressed in human ovarian cancer tissue specimens and cell lines. Loss-function-assays showed that knockdown of LINC00858 inhibited cell proliferation, migration and invasion of ovarian cancer cell lines. These findings suggested that LINC00858 might be an oncogene in ovarian cancer through increasing cell proliferation and migration.

LncRNAs exhibits the ability to act as a miRNA sponge, which acted as endogenous miRNA sponges by binding to miRNAs. As for the mechanism of LINC00858, it is known that LINC00858 exerts its roles via acting as a miRNA sponge. LINC00858 acts as a competitive endogenous RNA to sponge miR-3182 and then regulates MMP2 in lung cancer [12]. LINC00858 promotes the tumorigenesis of osteosarcoma through regulating the miR-139/CDK14 axis [14]. Our results proved that LINC00858 directly sponged miR-134-5p and regulated the expression of miR-134-5p in SKOV3 cells. Increasing evidence indicates that miR-134 is essential for tumorigenesis of multiple cancers including ovarian cancer [21, 22, 23]. MiR-134 participates in tumor cell proliferation, apoptosis, invasion, metastasis, drug resistance, as well as possesses potential roles in cancer diagnosis, treatment, and prognosis [24]. In the present study, we found that miR-134-5p was significantly lower in ovarian cancer cell lines. Inhibition of miR-134-5p prevented the tumor suppressive effects of LINC00858 knockdown, which further confirmed that LINC00858 executed its role via sponging miR-134-5p.

Currently, massive evidence suggests that miRNAs participate in a series of cellular processes via targeting multiple mRNAs [25, 26]. To assess the mechanism of miR-134-5p, online software was applied to predict the target genes. We found that miR-134-5p directly bound to the 3'UTR of TRIM44 and inhibited its expression. Moreover, TRIM44 was significantly up-regulated in ovarian cancer cell lines and mitigated the inhibitory effects of miR-134-5p on proliferation, migration and invasion of SKOV3 cells. These data reveal that TRIM44 might mediate the tumor suppressive effect of miR-134-5p in ovarian cancer. Consistent with our findings, TRIM44 acts as oncogene in several types of cancers, such as prostate cancer, non-small cell lung cancer, cervical cancer, and esophageal cancer [27, 28, 29, 30].

However, there are some limitations in this study. Firstly, only one ovarian cancer cell line SKOV3 was used to verify the effects of LINC00858 dysregulation on cell proliferation, migration and invasion *in vitro*. Secondly, the specific mechanism needs further investigation.

Conclusions

These findings demonstrated that LINC00858 exerted oncogenic role in ovarian cancer. Furthermore, the role of LINC00858 was mediated by miR-134-5p/TRIM44 axis (Fig. 6). Thus, LINC00858 might be a therapeutic target for ovarian cancer treatment.

Declarations

Authors' contributions

Ning Wang wrote this paper. Qin-Xue Cao and Lu Ren performed the experiments. Hai-Ling Cheng and Shao-Qin Yang interpreted and reviewed the experimental results. Jun Tian designed the study and analyzed the data.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was approved by the Ethic Committee of Huaihe Hospital of Henan University the Declaration of Helsinki Principles. Written informed consents were obtained from each participant involved in this study.

Competing interests

There was no conflict of interest.

Funding

None.

Acknowledgements

None.

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Figures

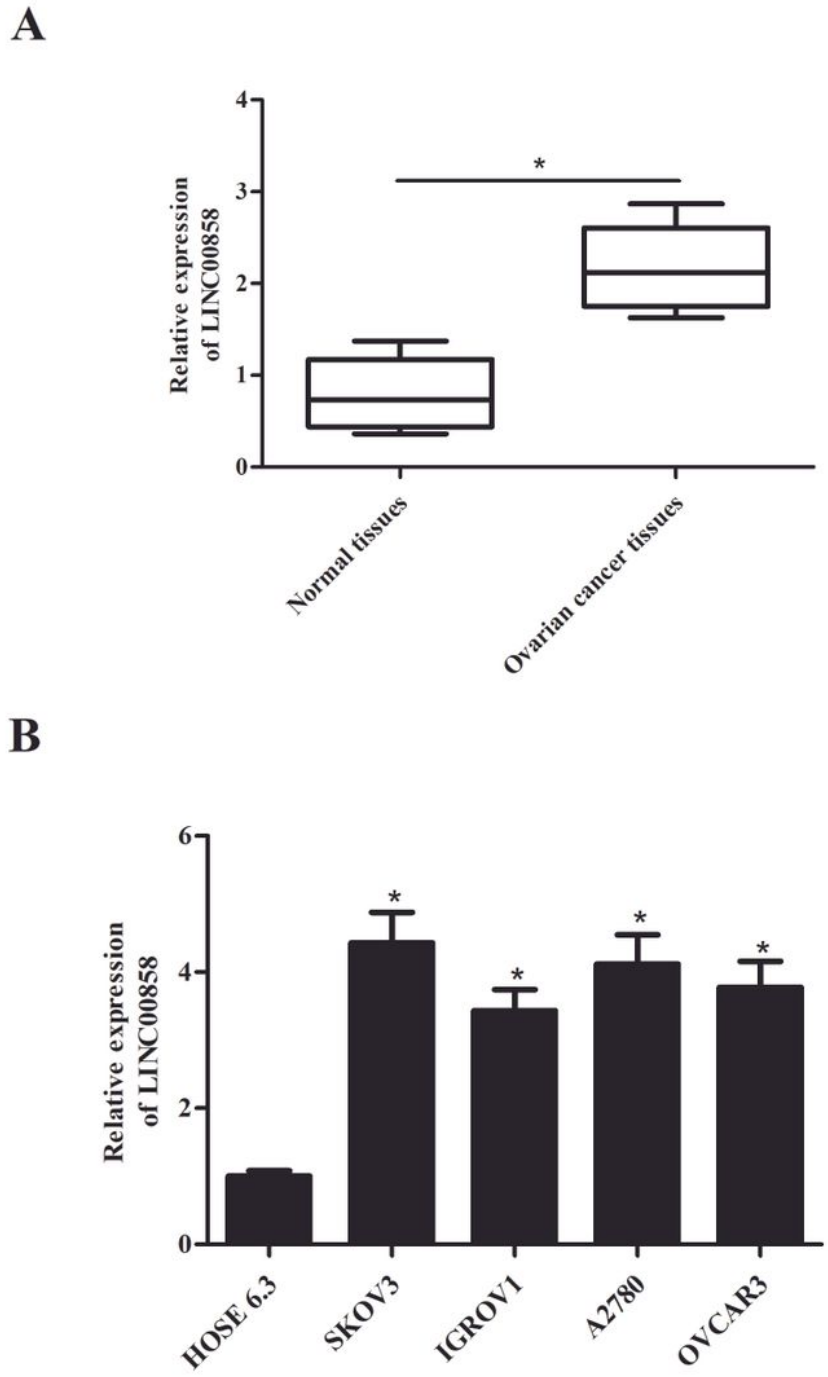

Figure 1

Up-regulated expression of LINC00858 in ovarian cancer tissues and cell lines. (A) Forty-six paired ovarian cancer tissue samples and non-tumor tissues were obtained to detect the LINC00858 expression by qRT-PCR method. *p<0.05 vs. non-tumor tissues. (B) LINC00858 expression was also detected in ovarian cancer cell lines (SKOV3, IGROV1, A2780, and OVCAR3 cells) and the normal cell line (HOSE 6.3 cells) using qRT-PCR. *p<0.05 vs. HOSE 6.3 cells.

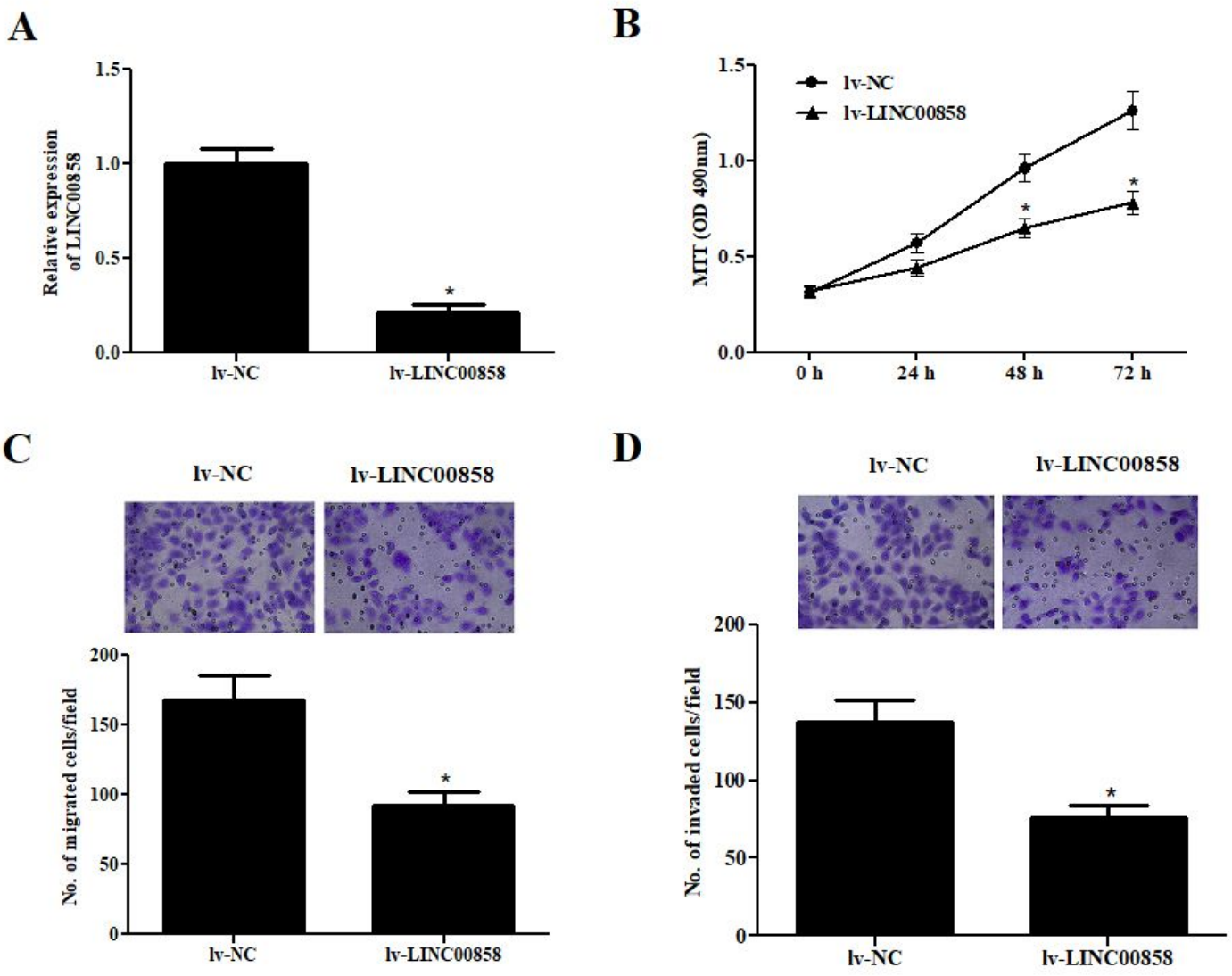

Figure 2

Loss-of-function assays were performed to evaluate the role of LINC00858 in the proliferation and invasion in SKOV3 cells. (A) A stable LINC00858-knockdown cells through infection with lv-LINC00858. The qRT-PCR was performed to detect the LINC00858 expression at 48 h post-infection. (B) Cell proliferation of SKOV3 cells was measured using MTT assay after infection with lv-LINC00858 or lv-NC. (C-D) Cell migration and invasion were detected by transwell assay. *p<0.05 vs. lv-NC-infected SKOV3 cells.

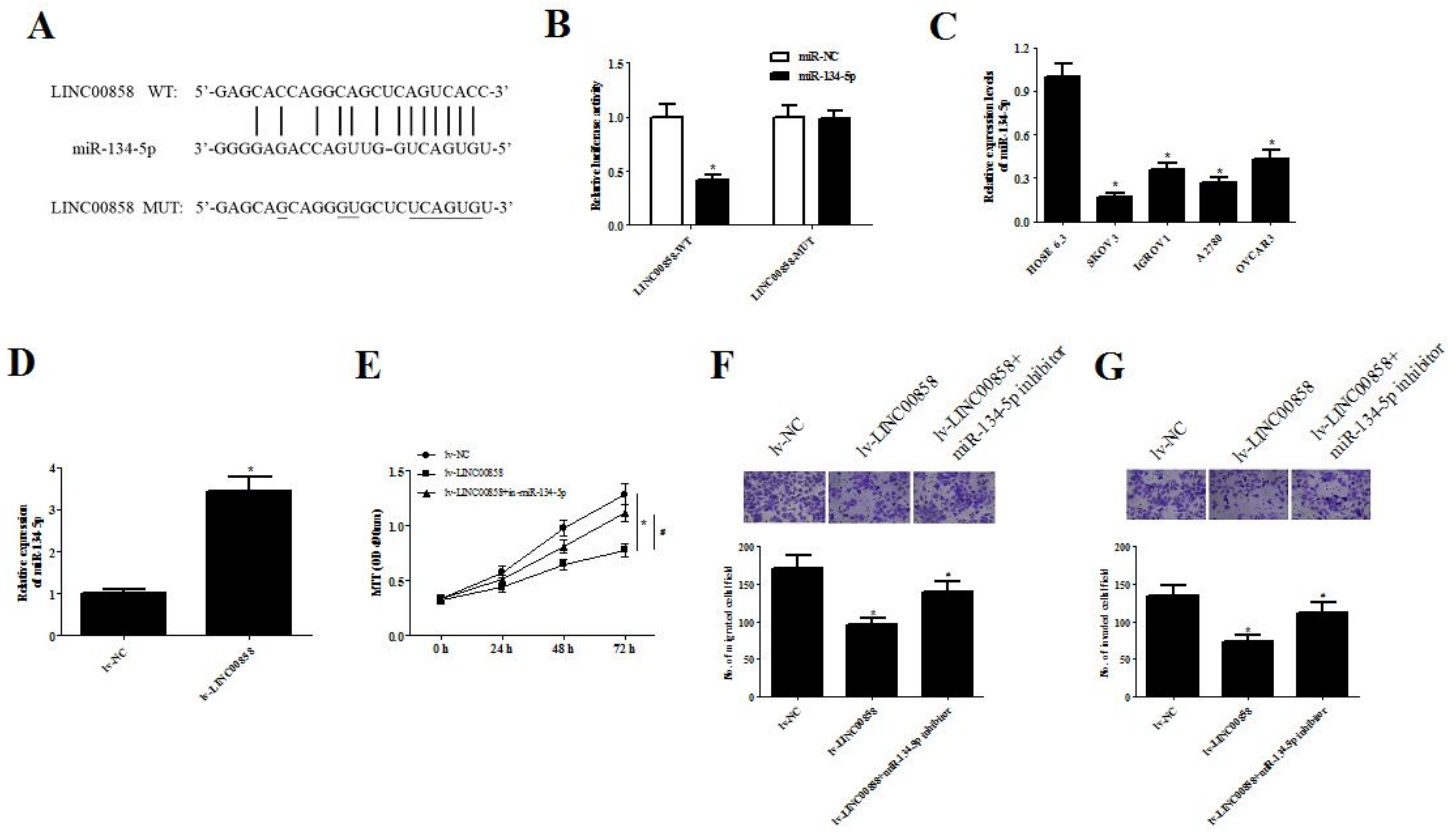

Figure 3

LINC00858 sponged miR-134-5p in SKOV3 cells. (A) Putative binding sequences between LINC00858 and miR-134-5p. (B) Luciferase activities of pmiRGLO-LINC00858-Wt and pmiRGLO-LINC00858-Mut plasmids after co-transfection with miR-134-5p mimics or control mimics. *p<0.05 vs. SKOV3 cells co-transfected with pmiRGLO-LINC00858-Mut and miR-134-5p mimics. (C) The expression of miR-134-5p was detected in ovarian cancer cell lines (SKOV3, IGROV1, A2780, and OVCAR3 cells) and normal cell line (HOSE 6.3 cells). *p<0.05 vs. HOSE 6.3 cells. (D) MiR-134-5p expression was detected using qRT-PCR after infection with lv-LINC00858 or lv-NC in SKOV3 cells. *p<0.05 vs. lv-NC-infected SKOV3 cells. (E-G) MTT and transwell assays were performed to detect proliferation, migration and invasion of SKOV3 cells after transfection with miR-134-5p inhibitor or control inhibitor. *p<0.05 vs. lv-NC group; #p<0.05 vs. lv-LINC00858 group.

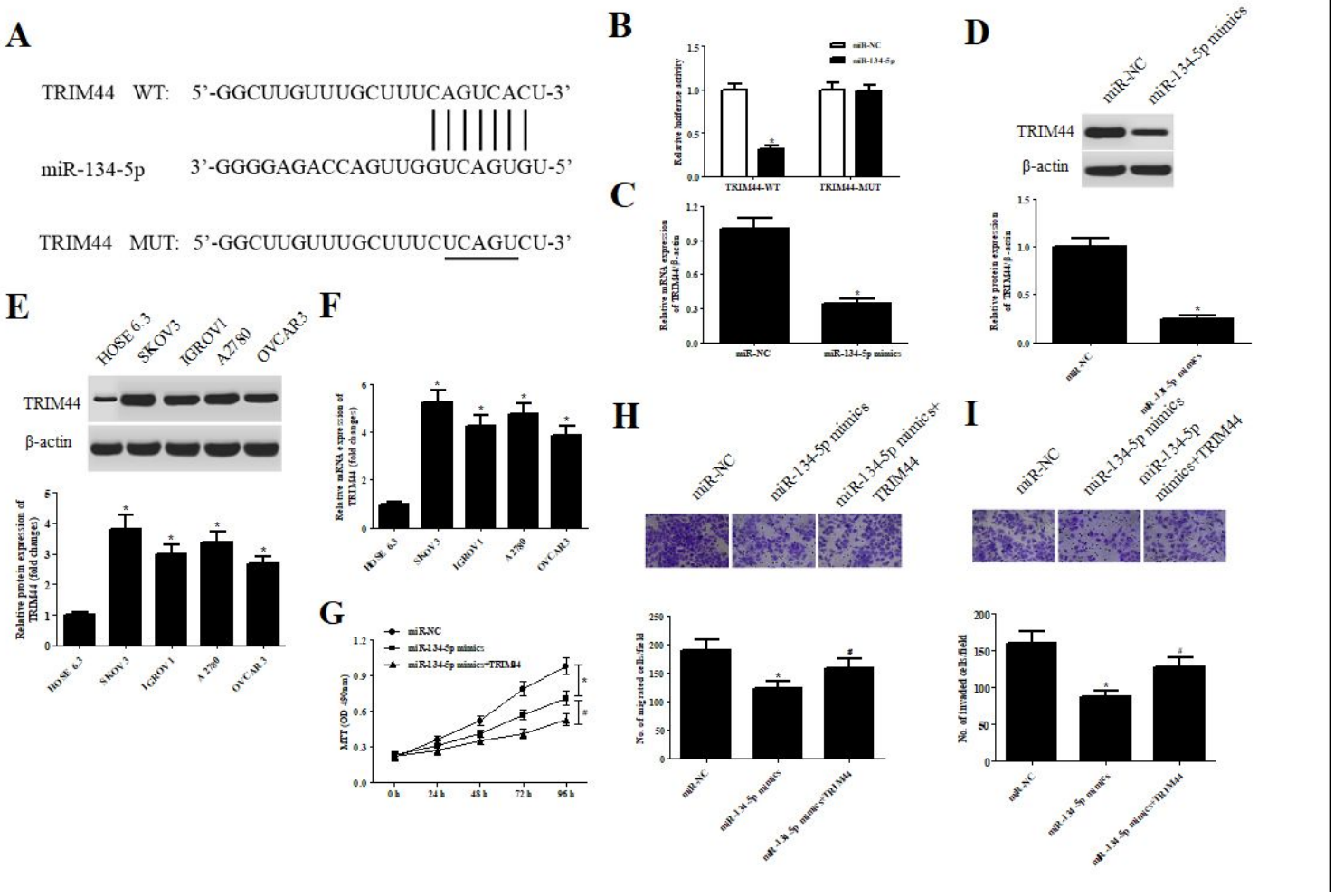

Figure 4

TRIM44 was a downstream target of miR-134-5p targeted TRIM44 in SKOV3 cells. (A) Putative miR-134-5p binding sequences in the 3'UTR of TRIM44. (B) Luciferase activities of pmiRGLO-TRIM44-Wt and pmiRGLO-TRIM44-Mut plasmids after co-transfection with miR-134-5p mimics or control mimics. *p<0.05 vs. SKOV3 cells co-transfected with pmiRGLO-TRIM44-Mut and miR-134-5p mimics. (C) TRIM44 expression was detected using qRT-PCR after transfection with miR-134-5p mimics or control mimics. *p<0.05 vs. control mimics-transfected SKOV3 cells. (D) TRIM44 expression was determined in ovarian cancer cell lines (SKOV3, IGROV1, A2780, and OVCAR3 cells) and normal cell line (HOSE 6.3 cells). *p<0.05 vs. HOSE 6.3 cells. (E and F) The protein and mRNA expression levels of TRIM44 were determined in ovarian cancer cell lines (SKOV3, IGROV1, A2780, and OVCAR3 cells) and normal cell line (HOSE 6.3 cells). *p<0.05 vs. HOSE 6.3 cells. (G-I) Effects of TRIM44 on cell proliferation, migration and invasion of SKOV3 cells were assessed using MTT and transwell assays after transfection with pcDNA3.1-TRIM44 or pcDNA3.1. *p<0.05 vs. miR-NC group; #p<0.05 vs. miR-134-5p mimics.

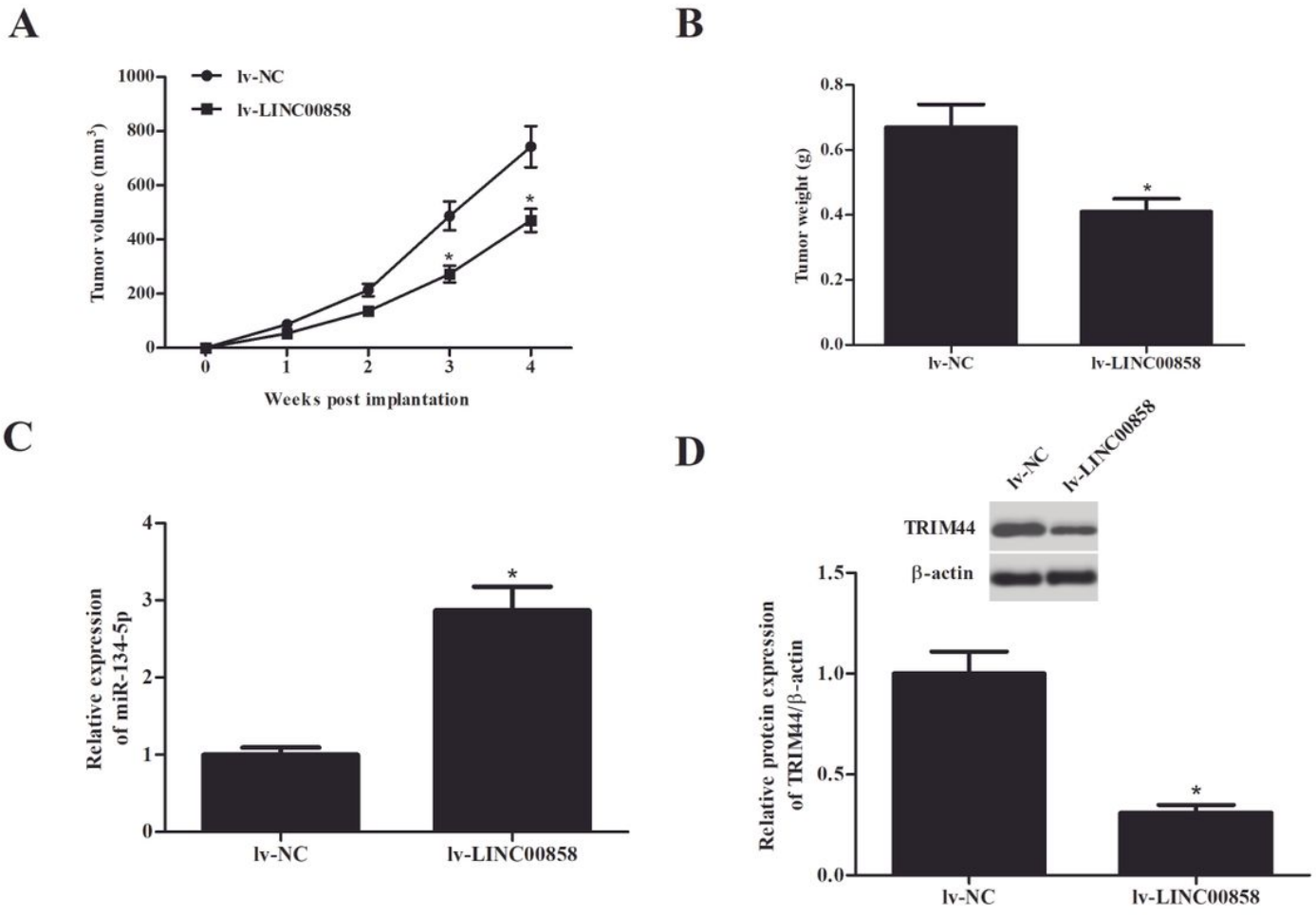

Figure 5

Tumor suppressive role of lv-LINC00858 in an in vivo xenograft model of nude mice. (A) Tumors were measured every 7 days after subcutaneously injection with lv-LINC00858 or lv-NC infected SKOV3 cells. (B) After four-weeks post injection, the mice were dissected and the tumors were collected and weighted. (C-D) Expression levels of miR-134-5p and TRIM44 were measured in the xenograft. *p<0.05 vs. mice injected with lv-NC-infected SKOV3 cells.

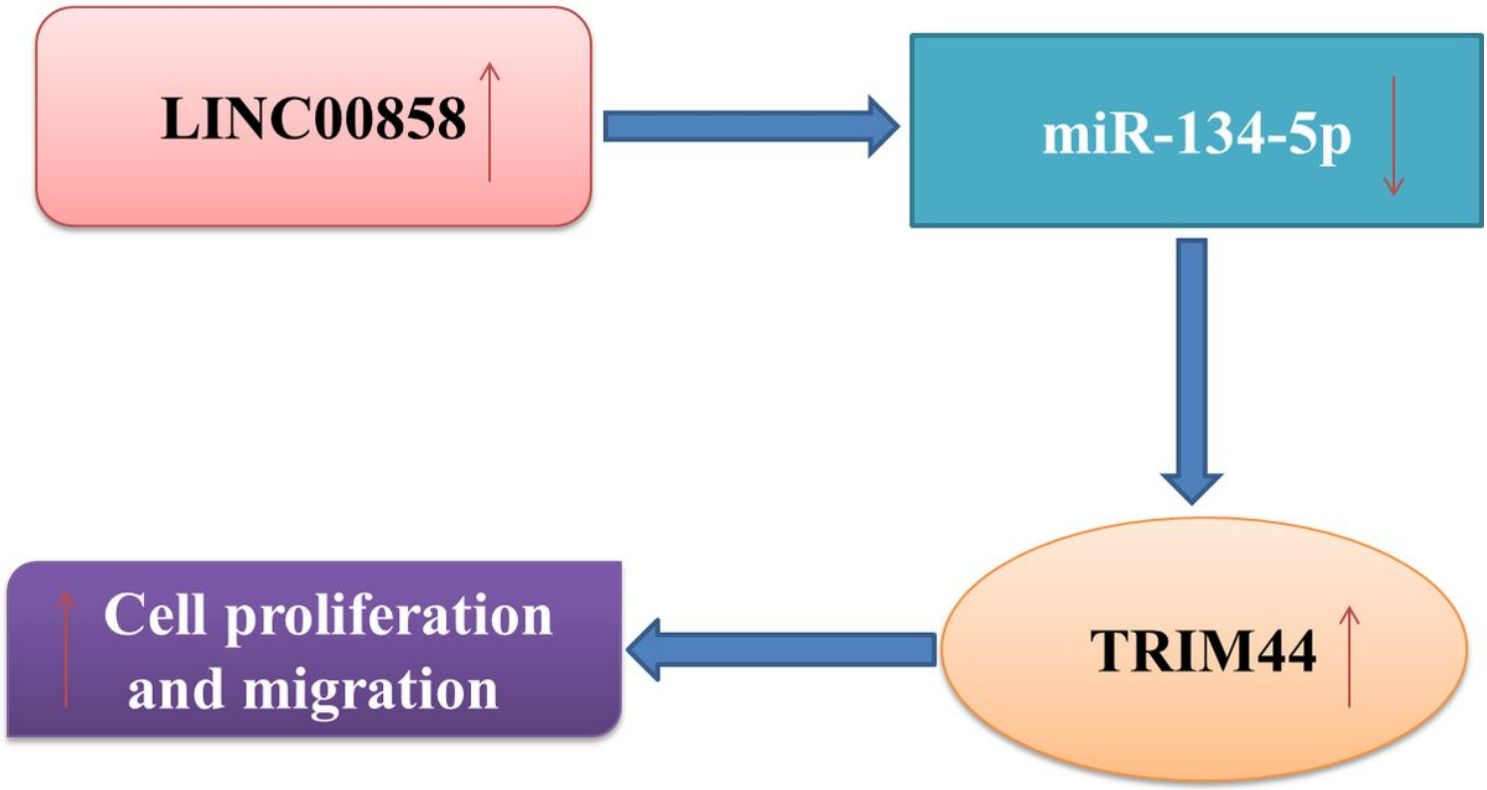

Figure 6

Diagram representation of LINC00858/miR-134-5p/TRIM44 axis in regulating the progression of ovarian cancer.