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Regulation of Transcription Factor YAP-TEAD by Non-coding RNA LINC00857 and the Inhibitory Effects on Ovarian Cancer Cell Proliferation

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ABSTRACT

This study was aimed to explore the expression and mechanism of the transcription factor YAP-TEAD in the Hippo signaling pathway under the regulation of non-coding Ribonucleic Acid (RNA) LINC00857 in the proliferation of ovarian cancer cells, so as to provide a scientific research basis for clinical diagnosis and treatment of ovarian cancer. In the study, the ovarian cancer cell lines (BT 549) were rolled into a control group (normal culture-defined as BT549/NC) and a response group (transfected with non-coding RNA LINC00857 cultured cells-defined as BT 549YAP cells). The expression and proliferation ability of the transcription factor YAP-TEAD in the two groups of cancer cells were analyzed and compared. The results showed that the YAP-TEAD expression rate was the highest in Bt549 cells; the YAP content grade (0.18) in BT 549-YAP cells was lower than BT 549/NC (0.2) after transfection ($P< 0.05$); and the apoptotic rate of the response group (80%) was higher than that of the control group (25%) after the intervention. With the extension of culture time, the expression of CCN1 mRNA decreased ($P< 0.05$), and CCN2 mRNA increased ($P< 0.05$). After 12, 24, 36, and 48 hours, the apoptosis rate of the reaction group at different time points was higher than that of the control group ($P< 0.01$). When YAP-TEAD was down-regulated, the in vitro proliferation ability of BT 549-YAP cells was weakened compared with BT 549/NC and parental cells. It was concluded that the non-coding RNA LINC00857 can target the transcription factor YAP-TEAD in the Hippo signaling pathway to decrease its expression, thus inhibiting the proliferation, migration, and invasion of cancer cells, and promoting cell apoptosis.

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Introduction

Ovarian cancer is a common gynecological disease (1), and an estimated 52000 individuals die from ovarian cancer each year worldwide (2). The fatality rate is the highest in Europe. In China, the incidence of ovarian cancer is relatively low, but due to the large population, there are 4.1 cases per 100,000 people. In 2019, there were 52,100 patients, among whom 2,250 died (3-5). Although there is much research on surgical methods and chemotherapy, in fact, there lacks progress in the treatment of ovarian cancer (6,7).

Studies have found that the Hippo signal transmission pathway (mainly divided into MST, LAT, and YAP) plays an important role in regulating the proliferation of ovarian cancer cells. It can sense and respond to mechanical signals to control the size, growth, and regeneration of tissue and organs (8). TEAD is a sensitive factor for ovarian cancer (9). HIP-TEAD can regulate ovarian function (10). In mammals, the main actuator molecule YAP/TAZ of the downstream hippo signaling pathway binds to the transcription factor TEAD (11). It acts on the downstream target gene CCN1/CYR61 (cysteine-rich 61) and other growth factors (CCN2, CTGF) to promote cell proliferation and differentiation and regulate cell adhesion, morphology, and polarity (12-17). However, the mechanism of the Hippo signaling mechanism regulating the proliferation of ovarian cells remains unclear.

RNA LINC00857 is a non-coding RNA with more than 300bp. Lacking an open leader frame, it cannot encode protein like mRNA, and it was held that RNA LINC00857 did not participate in genome transfer. However, recent studies have found that RNA

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linn00857 is involved in the expression, translation, and generation of protein (18-22).

There are reports that RNA LINN00857 can influence tumorigenesis through Hippo signaling pathway. What's more, RNA LINN00857 participates in the reproductive physiological processes, such as ovarian production, gamete maturation, and embryo production. It is used as a new biomarker for the risk prediction, early diagnosis, and prognosis determination of various tumors, such as prostate cancer, breast cancer, and cervical cancer thanks to its specificity, stability, and tracking ability (23-27). However, there are relatively few researches on the application of RNA LINN00857 in ovarian cancer. Therefore, in this study, ovarian cancer cells were used as the research subjects to explore the influence of RNA LINN00857 on the expression of YAP-TEAD. The influence mechanism of hippo signal transmission in cell proliferation was also discussed.

Materials and Methods

Reagents

Cancer cell culture medium from American Sigma; fetal bovine serum from American; Recombinant human leukemia inhibitor from American protein company; Epidermal Growth Factor (EGF) from a protein company in the United States; Basic Fibroblast Growth Factor (BFGF) from a protein company in the United States; Glial Cell line-derived Neurotrophic factor (GDNF) from a protein company in the United States; the penicillin mixed antibiotic from the protein company in the United States; the Sigma acrylate inhibitor of the United States; the protein A+G magnetic beads from Beijing Kangwei Century Company; and the high-sensitivity chemiluminescence detection kit protein printing membrane regeneration solution from Beijing Kangwei.

Grouping and transfection

4 kinds of ovarian cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-468, and BT 549) (purchased from the Chinese Academy of Sciences (Shanghai, China)) were selected, among which YAP-TEAD had high expression activity as a research sample. Then, the ovarian cancer cells were rolled into a control group and a response group. The ovarian cancer cell lines in the response group were transfected with non-coding RNA LINC00857 cultured cells, and the transfected cells can be defined as BT-549YAP cells. In the control group, ovarian cancer cells were cultured normally and defined as BT549/NC. In the logarithmic propagation stage, cells underwent a morphological transformation at a confluence of about 30% to 50%. Libufedaminic acid was used as a carrier for the trans reaction. The used reagents and articles were disinfected. The trans-complex was added to the culture medium, and the liquid was then transferred to the 6-well plate, with 2mL in each glass dish. Next, the cells were incubated at 37°C under 5% CO_2. The RT-qPCR was performed 2-3 days LATER.

Extraction of total RNA

1mL of Trisol was added to 50-100 mg of ovarian cancer cells, followed by blowing to lyse cells. The liquid was then transferred to a 1.5mL centrifuge tube and let still at room temperature for 5 minutes. Subsequently, 0.5mL of isopropanol was added, followed by centrifugation at 12000rpm for 10 minutes at room temperature, and then, the supernatant was discarded. Next, 1mL of 75% ethanol was added, followed by centrifugation at 750rpm for 5 minutes, and the supernatant was discarded. The RNA precipitate was then dried to translucent, and seamless water was added accordingly.

Reverse transcription

2mg RNA separately was immersed in 1.2mL of 10mg Oligo(dT), and Rnase-free Ovate was added until the volume reached 15mL, followed by a water bath for 5 min (70°C). Immediately, the liquid was iced for 5 min. Subsequently, 5 XM-MLV for reverse transcription was added, followed by a water bath for 1 h (42°C). Next, the liquid was stored at -80°C for later use. At that time, RNase-free water was used to dilute the cDNA solution to 100mL.

Primer design and synthesis

GenBank was searched for published gene sequences. Primer Express software (Perkin Elmer) was used to design and amplify real-time quantitative PCR primers for YAP, CCN1, CCN2, and internal reference GAPDH genes, and Shanghai Environmental Protection Technology Company was responsible for synthesis.

YAP primer sequence:
FORWARD: 5'-GGAACGAGACGAGGAGATGG-3'
REVERSE: 5'-AGGATGTGGAACCTGTAGAGC-3'
CCN1 primer sequence:
FORWARD: 5'-TAGGTCATCTTCCTCACCTG-3'
REVERSE: 5'-GAGGTCATCTTCCTCACGAG-3'
CCN2 primer sequence:
FORWARD: 5'-AAGCTTACGCCATTTTGACCCAGAG-3'
FORWARD: 5'-AAGGTACCAAGTGTGCCTGAAAAAT-3'

Quantitative detection of target gene mRNA

Sequence analysis: PCR products were acquired after TA cloning.

Fluorescent quantitative PCR reaction system (20mL): the reaction lasted for 2 minutes at 95°C, and the baseline CT value was adjusted according to the fluorescent signal collected to obtain the CT value of the sample. The 2-PCT method was used to calculate the relative cell expression.

Western blot

Cell lysates were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, USA), and washed with RIPA lysate (Shanghai Biyuntian). The specific primary antibody ((Shanghai Miaohui Biotechnology Co., Ltd., Shanghai, China)) was adopted for immunostaining. Chemiluminescence detection was performed by using the Pierce ECL Western Blotting Substrate (Thermo Scientific). The fluorescence signals were collected using Image Lab software (v4.1), and the quantification of western blots was analyzed using Image J version 1.48.

CCK8 cell viability assay

CCK8 reduction was used to evaluate cell viability and simultaneously quantify reductase expression/activity of cells upon drug incubation as described elsewhere. In brief, 100 μL of cell solution containing 3×10^4 cells were placed in a 96-well plate at a concentration of 24-h incubation with drugs. CCK8 was added to each well to a final concentration of 0.8 mg/mL. Absorbance was measured at 610 nm on a plate reader to quantify the reduction of CCK8. All measurements were done in triplicates and normalized to DMSO controls.

Flow cytometry

Ovarian cancer cells were collected first. 48 hours after the reaction, the liquid was transferred to a tube for centrifugation, and the supernatant was discarded. The cells were then digested by trypsin for 30 min at 37 °C, followed by centrifugation again. After washing, cells were suspended in ice-cold staining buffer (sterile PBS containing 2% FBS). Cells were blocked with anti-CD16/32 on ice for 10 min. After blocking, 1×10^6 cells were suspended in 0.1 mL of ice-cold staining antibody buffer and stained on ice in the dark for 20 min. After washing twice in staining buffer, samples were acquired on a Cytek Aurora flow cytometer. Results were analyzed using FCS Express 6 flow cytometry software (De Novo Software).

Colony formation assay

A colony formation assay was performed to test the cell proliferation ability. Cells were seeded in 12-well plates (600 cells/well for MDA-MB-231; 1500 cells/well for MCF-7; 500 cells/well for MCF-10A). The cells were treated with SPA, LM-2I, or vehicles, and the medium was replaced every 3 days until visible colonies were formed. At the end of the experiment, cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet in 20% ethanol for 30 min.

Statistics

Each experiment was repeated more than three times, and Graphpad Prism 5.0 was used for statistical analysis. All data were expressed as mean±SEM, and one-way ANOVA analysis and multiple comparison analysis (Tukey's multiple comparison test) were performed. $P<0.05$ was the threshold for significance.

Results and discussion

The expression of YAP-TEAD in different kinds of ovarian cancer cells

The expression levels of YAP-TEAD in 4 kinds of ovarian cancer cells (MCF-7, MDA-MB-231, MDA-MB-468, BT 549) were identified using the RT-qPCR method, as shown in Figure 1. YAP-TEAD was highly expressed in cancer cell lines, but YAP-TEAD had the highest expression rate in BT 549 cells, so BT 549 cells served as the target cells.BT-549 cells are from papillary invasive duct tumor tissue, and thus BT-549 cells are polymorphic, including epithelial-like components and multinucleated giant cells, which can be used to study the proliferation and metastasis of cancer cells.

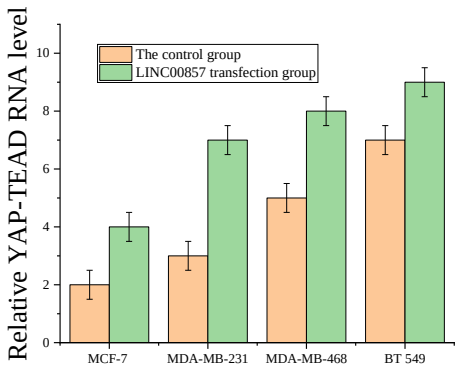

Figure 1. The expression of YAP-TEAD

The influence of non-coding RNA LINC00857 transfection on the expression of YAP-TEAD

To study the influence of non-coding RNA LINC00857 transfection on the expression of YAP-TEAD in BT 549 cells, the expression levels before and after transfection were detected.YAP-TEAD had the highest expression rate in BT 549 cells among the four kinds of cells, so BT 549 cells served as the target cells. In the response group, the cells were cultured with non-coding RNA LINC00857 for transfection, and the transfected cells were defined as BT 549YAP cells. In the control group, the cells were cultured normally, defined as BT 549/NC. Experiments have found that the transfection efficiency of BT 549 can reach more than 90% with MOI=45. Then, the expression of YAP-TEAD before and after transfection was analyzed. Both RT qPCR and Western blot results showed that YAP-TEAD expression was down-regulated in BT 549YAP cells, with GAPDH used as an internal reference. The survival of neurons depends on YAP, and the content of YAP is different in different cells. In BT 549-YAP cells, the content grade of YAP was 0.18, while in BT 549/NCcells, it was 0.2, showing significant differences, $P<0.05$. It suggested that YAP-TEAD expression was down-regulated in BT 549YAP cells transfected by non-coding RNA LINC00857, as shown in Figure 2.

The effect of down-regulated YAP-TEAD expression on cell apoptosis

It was known that the expression of YAP-TEAD was down-regulated in BT 549YAP cells transfected by non-coding RNA LINC00857 in the response group versus the control group. To study the effect of down-regulated YAP-TEAD expression on the apoptosis of BT-549 cells, the cell apoptosis in the two groups was analyzed. The results showed that, in the response group, before transfection, the number of BT-549 cells was between 2.0×10^3-6.0×10^3, while after transfection, the number of BT-549 cells was within 2.0×10^3. As for the comparison between groups, there was no statistically significant difference in the number of BT-549 cells between the response group and the control group before the intervention; while after the intervention, the apoptosis rate in the response group was as high as 80%, while it was only 25% in the control group, indicating that the down-regulated expression of YAP-TEAD inhibited the proliferation of cancer cells, as shown in Figure 3 and Figure 4.

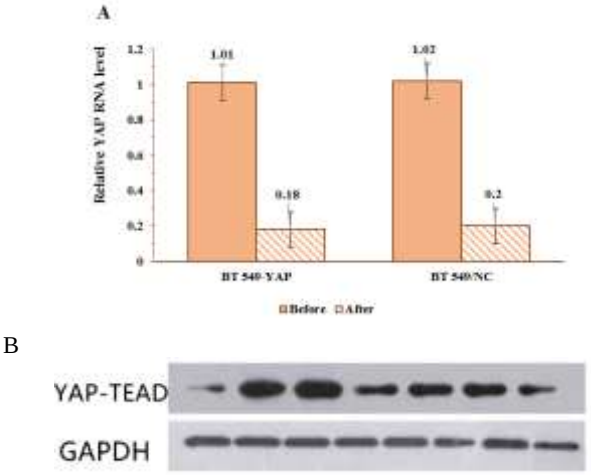

Figure 2. The expression of YAP-TEAD before and after transfection

Expression of CCN1 and CCN2 under different culture time

When entering the nucleus, YAP binds to TEAD, regulates target genes CCN1, CCN2, and then regulates biological processes, such as cell proliferation and apoptosis. To further study the mechanism of the Hippo signaling pathway in regulating the proliferation of ovarian cancer cells, we isolated and cultured ovarian cancer cells for 0, 6, 12, and 24 hours, and used Real-time PCR to detect the mRNA expression of CCN1 and CCN2. It was found that the expression of CCN1 mRNA was decreased with the increase in culture time ($P<0.05$), while the expression of CCN2 mRNA was increased when the cells were cultured for 6 hours ($P<0.05$), but

decreased when the cells were cultured for 12 hours and 24hours (P<0.05), indicating the Hippo signaling pathway regulates the proliferation of cancer cells by acting on target genes (Figures 5).

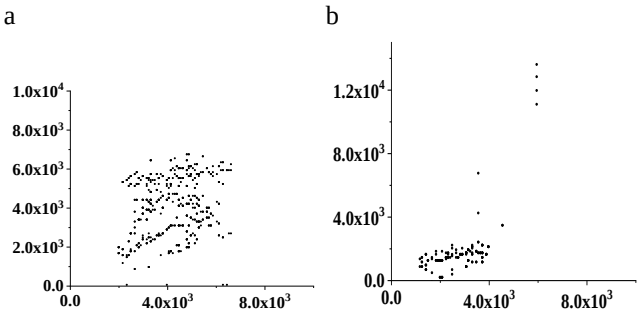

Figure 3. (a) Cell content before transfection (b) Cell content after transfection

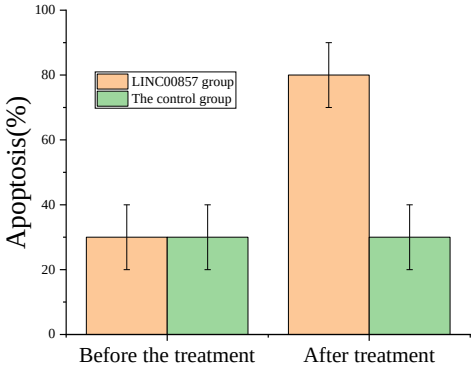

Figure 4. Apoptosis rate before and after the intervention

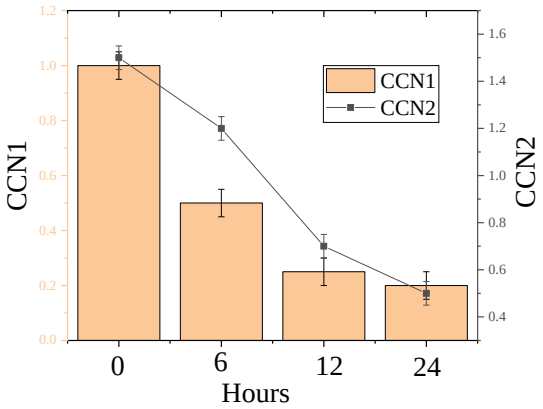

Figure 5. Expression of CCN1 and CCN2 expression under different culture times

The effects of non-coding RNA LINC00857 on the proliferation of ovarian cancer cells

To identify the effects of non-coding RNA LINC00857 on cell proliferation, the cell apoptosis rate of the response group and the control group was determined by CCK8. Figure 6 showed apoptosis rates at different time points in the two groups. After 12, 24, 36, and 48 hours, the apoptosis rates of the response group were higher versus the control group at different time points, and the difference was notable (P<0.01). It indicated that RNA LINC00857 significantly inhibited the proliferation of cancer cells, and there was a statistically significant difference versus the control group (P<0.01).

The effect of down-regulated YAP-TEAD expression on cell proliferation

CCK8 method was used to detect the in vitro proliferation ability of BT-549YAP, BT-549/NC, and parental cells, and the growth curve was drawn. As shown in Figure 8, compared with BT 549/NC and parental cells, the in vitro proliferation ability of BT 549-YAP cells was weakened. Figure 7 demonstrated that compared with the BT-549/NC, the clonogenic ability of BT-549YAP cells was weakened, suggesting that the BT-549YAP cells with down-regulated YAP-TEAD expression exhibited weakened proliferation ability versus the BT 549/NC and parental cells.

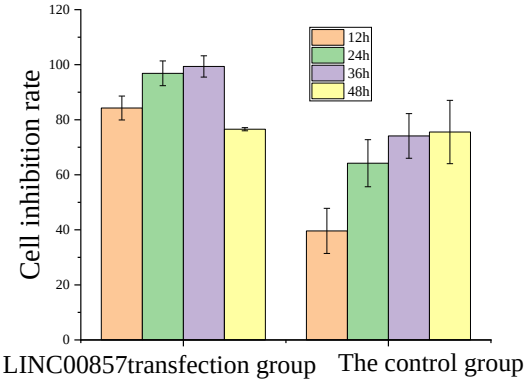

Figure 6. The apoptosis rates at different time points

The American Cancer Society reveals that most patients with ovarian cancer are diagnosed in the advanced stage (28). Intra-abdominal metastasis, as the main feature of progressive ovarian cancer, leads to a high mortality rate of the disease to a certain extent. At the beginning of treatment, most patients are sensitive to chemotherapy for primary or secondary tumor cells. In view of the dissatisfying therapeutic effects, developing an alternative drug for ovarian cancer is the priority.

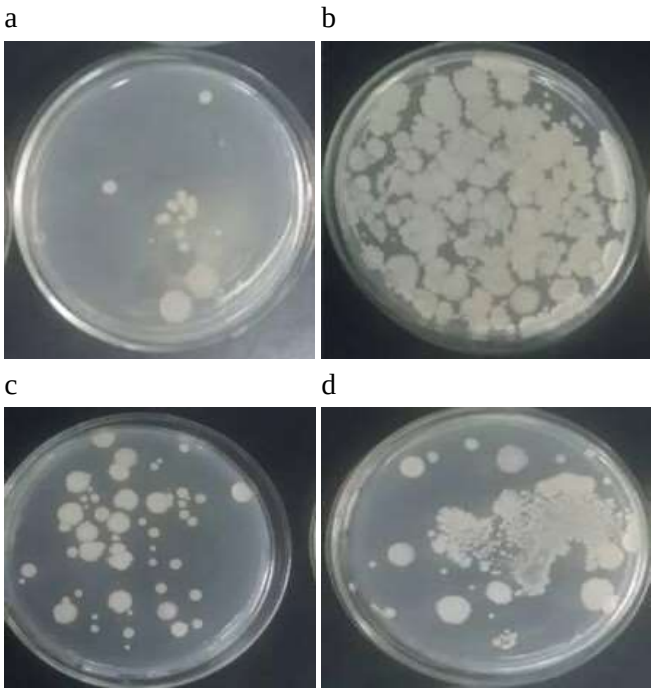

Figure 7. (a) The control group before cultured (b) The control group after cultured (c) The response group before cultured (d) The response group after cultured

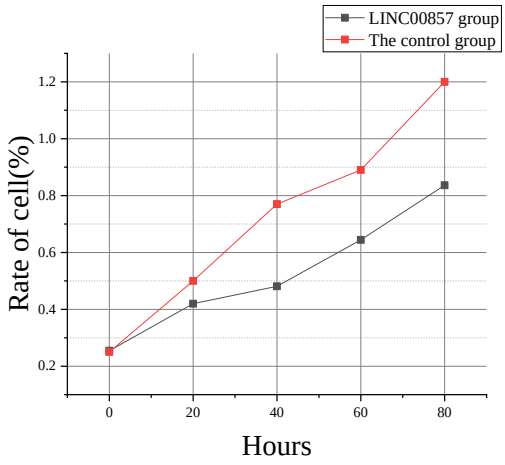

Figure 8. Growth curve of in vitro proliferation ability

Studies have shown that YAP, the transcription core of the HPPPO signal transmission pathway, is highly expressed in ovarian cancer tissue. At the same time, the high activity of YAP can promote the proliferation of ovarian cancer cells, inhibit apoptosis caused by amino acids, and enhance cell movement and non-adhesive growth. Therefore, YAP is considered an important oncogene for ovarian cancer. Studies have found that the Hippo signal transmission path is the main factor contributing to embryo production (29). With the activation of YAP, the expression of target genes in the Hippo signaling pathway is increased significantly. Since the coupling of YAP and TEAD 4 in the CNP and CN2 is stable, CNP and CN2 are considered the direct transfer targets of the YAP/TEAD complex.Hippo/YAP signaling pathway can regulate organ activity by promoting cell proliferation and inducing cell apoptosis (30). Subsequent research shows that the Hippo signal transmission path is an important one for the development of ovarian cancer(31).

In the study, it was found that YAP-TEAD was highly expressed in cancer cell lines, while YAP-TEAD had the highest expression rate in BT 549 cells, so BT 549 cells served as the target cells. Such results are consistent with the research results of Pederson et al. (2020) (32). In subsequent studies, it was concluded that when MOI = 45, the transfection efficiency of BT 549 can reach more than 90%. After the results of RT-qPCR and Western blot were transfected with non-coding RNA LINC00857, the expression of YAP-TEAD in BT 549YAP cells was down-regulated (GAPDH was used as an internal reference). Crawford et al. (2018) (33) proposed that the activation or overexpression of the YAP-TEAD transcriptional co-activator had been shown to cause cell transformation and tumor development. Therefore, this study analyzed the down-regulation of YAP-TEAD expression and the apoptosis of BT-549 cells. The results showed that before the intervention, there was no statistically significant difference in the number of BT-549 cells between the response group and the control group (P> 0.05); and after the intervention, the apoptosis rate of the response group was as high as 80%, while that of the control group was only 25%. It shows that the down-regulation of YAP-TEAD expression promotes the apoptosis of cancer cells. Many studies have also shown that the decreased activity of the YAP-TEAD factor in the Hippo pathway can inhibit the growth of cancer cells (34, 35), which is consistent with the results of this study. Through the analysis of the mechanism of the Hippo signaling pathway regulating the proliferation of ovarian cancer cells, it was found that the expression of CCN1 mRNA decreased with the extension of culture time (P< 0.05), while the expression of CCN2 mRNA increased with the extension of culture time (P< 0.05). However, it decreased with the extension of culture time (P< 0.05), indicating that the Hippo signaling pathway

regulates the proliferation of cancer cells by acting on target genes. Another study suggested that CCN1 has the effect of promoting YAP-TEAD expression activity, which is opposite to the effect of CCN2 (36, 37).To prove the inhibitory effects of non-coding RNA LINC00857 on cell proliferation, the cell apoptosis rate of the response group and the control group was determined byCCK8. It was found that After 12, 24, 36, and 48 hours, the apoptosis rates of the response group were higher versus the control group, and the difference was notable (P<0.01). To explore the effect of down-regulated YAP-TEAD expression on cell proliferation, the CCK8 method was used to detect the in vitro proliferation ability of BT-549YAP, BT-549/NC, and parental cells, and the growth curve was drawn. It was found that, compared with BT 549/NC and parental cells, the in vitro proliferation ability of BT 549-YAP cells was weakened, compared with the control group, the clonogenic ability of BT-549YAP cells was weakened, suggesting that, the transfected cells with down-regulated YAP-TEAD expression exhibited weakened proliferation ability versus the BT 549/NC and parental cells. This is consistent with the conclusions of Wang et al. (2020) (38), indicating that this study has a certain degree of accuracy.

Conclusions

In the study, the role of the Hippo-YAP signal pathway in the proliferation of ovarian cancer cells was explored, and then the influence of non-coding RNA LINC00857 transfection on the expression of YAP-TEAD was investigated. It was found that Hippo/YAP signaling pathway can regulate organ activity by promoting cell proliferation and inducing cell apoptosis. YAP-TEAD expression was down-regulated in BT 549YAP cells transfected by non-coding RNA LINC00857, and the down-regulated expression of YAP-TEAD inhibited the proliferation of cancer cells. However, some limitations should be noted in the study.In this study, the LINC00857 antagonist was not used for negative control experiments. As far as the regulatory function of LINC00857 was concerned, there was a lack of strong comparison. It will pay attention to this in future studies. The study focuses on the influence of non-coding RNA LINC00857 transfection on the expression of YAP-TEAD, and the specific mechanism of the Hippo-YAP signal pathway in the proliferation of ovarian cancer cells needs further exploration. In conclusion, the study gives a new direction for the treatment of ovarian cancer.

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Conflict interest

None.

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