

LBX2-AS1 Promotes Ovarian Cancer Progression by Facilitating E2F2 Gene Expression via MIR-455-5P and MIR-491-5P Sponging

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Research

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Abstract

Background:LBX2-AS1 is a long noncoding RNA that facilitates the development of gastrointestinal cancers and lung cancer, but its participation in ovarian cancer development remained uninvestigated.

Methods: Clinical data retrieved from TCGA ovarian cancer database and the clinography of 60 ovarian cancer patients who received anti-cancer treatment in our facility were analyzed. The overall cell growth, colony formation, migration, invasion, apoptosis and tumor formation on nude mice of ovarian cancer cells were evaluated before and after lentiviral-based LBX2-AS1 knockdown. ENCORI platform was used to explore LBX2-AS1-interacting microRNAs and target genes of the candidate microRNAs. Luciferase reporter gene assay and RNA-pulldown assay were used to verify the putative miRNA-RNA interactions.

Results: Ovarian cancer tissue specimens showed significant higher LBX2-AS1 expression levels than non-cancerous counterparts. High expression level of LBX2-AS1 significantly associated with patients reduced overall survival. LBX2-AS1 knockdown significantly downregulated the cell growth, colony formation, migration, invasion and tumor formation capacity of ovarian cancer cells and increased their apoptosis in vitro. LBX2-AS1 interacts with and thus inhibits the function of miR-455-5p and miR-491-5p, both of which restrained the expression of E2F2 gene in ovarian cancer cells via mRNA targeting. Transfection of miRNA inhibitors of these two miRNAs or forced expression of E2F2 counteracted the effect of LBX2-AS1 knockdown on ovarian cancer cells.

Conclusions

LBX2-AS1 was a novel cancer-promoting lncRNA in ovarian cancer. This lncRNA increased the cell growth, survival, migration, invasion and tumor formation of ovarian cancer cells by inhibiting miR-455-5p and miR-491-5p, thus liberating the expression of E2F2 cancer-promoting gene.

Background

Ovarian cancer is the second most frequently diagnosed gynecological cancer and the leading cause of gynecological cancer-related death [1]. Common treatment strategies include surgery and adjuvant chemotherapy, but because its often diagnosed as late stage, patients' outcome remains to be improved. The pivotal roles played by long noncoding RNAs (lncRNAs) in the development of ovarian cancers have been increasingly addressed[2-3]. LncRNAs are known to regulate gene expressions by "sponging" microRNAs, thus participating in the cancer development[4-5]. MicroRNAs (miRNAs) are small RNAs (< 200 nt) that are known to repress the expression of their target genes by interacting with their binding sites on the target gene mRNAs and suppressing protein translation[6], and lncRNAs could regulate the expression of miRNA target genes by competitively binding to these miRNAs[7]. LncRNAs including FLVCR1-AS1, HOXD-AS1, MLK7-AS1 and HOTAIR[8-11] have been reported to promote ovarian cancer development through such mechanisms, while some other lncRNAs, such as MAGI2-AS3 [12], showed a cancer-suppressive role in ovarian cancer development.

lncRNA LBX2-AS1 has been previously found as a cancer-promoting lncRNA in several types of cancers mainly by sponging cancer suppressive miRNAs [13-14], but its role and mechanism of action in the development of ovarian cancer remain undetermined. In this research, we found that LBX2-AS1 expression level was significantly increased in ovarian cancer, in association with patients' reduced overall survival; knockdown of this lncRNA could significantly reduce the cell growth, colony formation, migration, invasion and tumor formation of the ovarian cancer cells and increase their apoptosis. We also found that LBX2-AS1 could directly binds to and inhibit the function of miR-455-5p and miR-491-5p, both of which restrained the expression of E2F2 by interacting with the 3' UTR of its mRNA. Our data showed that inhibition of these two miRNAs or the forced expression of E2F2 counteracted the effect of LBX2-AS1 knockdown on the malignancy of ovarian cancer cells.

Materials And Methods

Collection of patient-derived tissue specimens and meta-analysis

A total of 60 ovarian tissues and paired adjacent nontumor tissues were collected from patients who received surgical resection at Nanjing Maternity and Child Health Care Hospital. None of the patients had undergone chemotherapy or radiotherapy prior to surgery. Following surgical resection, all tissues were immediately frozen in liquid nitrogen and stored at -80°C until use for total RNA isolation. The present study was approved by the Ethics Committee of Nanjing Maternity and Child Health Care Hospital and was performed in accordance with the Declaration of Helsinki. Written informed consent was provided by all participants before inclusion in the study.

TCGA ovarian cancer dataset were analyzed using UCSC Xena TCGA data browser (<https://xena.ucsc.edu>). Clinical data were retrieved from the medical records of 60 ovarian cancer patients who received anti-cancer treatment in our facility during 2015-2018. The patients were at the age of 47 ± 5.12 , who were diagnosed with the tumor at stage II and received adjuvant chemotherapy after radical tumorectomy. Informed consent in written was obtained from each patient. The patient-derived ovarian cancer tissue specimens and the contralateral, non-cancerous ovarian tissue specimens were obtained during surgery and were stored in liquid nitrogen before analysis. A total of 1657 and 1436 cases from the Kaplan-Meier Plotter Database were collected to analyze overall survival and progression-free survival rates.

Cell culture and and stable cell line selection

SKOV3, OVCAR-3, Caov-3 and ES-2 human ovarian cancer cell lines as well as the human ovarian surface epithelial cells (HOSEPICs) were purchased from the cell bank of the Chinese academy of science (Shanghai, China). These cells were cultured in a humidified atmosphere at 37 degree with 5% CO_2 using RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% of pen/strep stock solution purchased from Thermo Fisher Scientific (Shanghai, China)[15]. LBX2-AS1 knockdown in vitro was achieved by lentiviral-based delivery of two different shRNA (sh-LBX2-AS1#1 and sh-LBX2-AS1#2) into

the target cells. A non-targeting shRNA (sh-NC) was used as negative control for LBX2-AS1 knockdown. The lentiviral particles were commercially purchased (Genecopoeia) and were used at a titer of 10^6 U/ml using Lipofectamin 2000 (Thermo Fisher Scientific) as the transcription reagent at a ratio of 1:20 in antibiotic-free cell culture medium. The cells were selected with Puromycin (Thermo Fisher Scientific) 48 hours after transfection for two passages. The efficiency of LBX2-AS1 knockdown was evaluated by qRT-PCR. Augmentation and inhibition of miR-455-5p or miR-491-5p were achieved by transfecting commercially purchased miR-455-5p or miR-491-5p mimic and inhibitor (Ribobio, Guangzhou, China), respectively, at a concentration of 100nM into the target cells using Lipofectamin 2000 (Thermo Fisher Scientific) as the transcription reagent at a ratio of 1:20 in antibiotic-free cell culture medium. Cells were analyzed 24 hours after transfection.

Evaluation of RNA expression levels

Total RNA (>200nt) or total small RNA (<200nt) were extracted using spin column-based isolation kit (Beyotime, Shanghai, China). The cells cultured in vitro and the patient-derived tissue specimens were first lysed using the lysis buffer associated with the kit before RNA extraction. The RNA extraction products were subject to qRT-PCR using commercially purchased cDNA synthesis kit, primers and qPCR mix (Genecopoeia, Guangzhou, China). For RNA >200nt, GAPDH was used as housekeeping gene, and RNU6B was used as housekeeping gene for small RNA. Primers used in this research were as follows: LBX2-AS1 Fw: 5'-AGTTTGTCCAGGTTTGGCA-3'; LBX2-AS1 Rev: 5'-CATGCCAGGGTCCTTGTTCT-3'; E2F2 Fw: 5'-GAGCTCACTCAGACCCCAAG-3'; E2F2 Rev: 5'-AACAGGCTGAAGCCAAAAGA-3'; GAPDH Fw: 5'-CCACATCGCTCAGACACCAT-3'; GAPDH Rev: 5'-TGACAAGCTTCCCGTTCTCA-3'; miR-455-5p Fw: 5'-GCCGCCTATGTGCCTTTGGACT-3'; miR-491-5p Fw: 5'-GGAGTGGGGAACCCTTCC-3'; RNU6B Fw: 5'-CTCGCTTCGGCAGCACA-3'; RNU6B Rev: 5'-AACGCTTACGAATTTGCGT-3'. The reverse primer for the two miRNAs were universal miRNA primer associated with the qPCR kit.

Cell proliferation

The overall cell growth of the SKOV3 and OVCAR-3 ovarian cancer cells was evaluated by CCK-8 cell proliferation assay. Briefly, the cells were seeded on 96-well-plate at a 1000 cells/well ratio; after 24, 48 or 72 hours of cell culture, the cells were incubated with CCK-8 solution at a ratio of 1:10 in the cell culture medium for 1 hour, and the amount of cells in each well was compared by measuring the OD 450 value of each well using a microplate reader.

Cell colony formation assay

The colony forming ability of the SKOV3 and OVCAR-3 cells was evaluated by clone formation assay. Briefly, 200 cells resuspended in 10 ml cell culture medium were cultured in a 100 mm petri dish. 2 weeks after cell culture, the clones on the petri dish were fixed with 4% paraformaldehyde, followed by staining with GIMSA solution (Sigma-Aldrich, Shanghai, China). The clones on each petri dish were counted in 3 randomly picked view under microscope.

Transwell invasion assay

Invasiveness and mobility of the SKOV3 and OVCAR-3 cells were evaluated by transwell assay using 8 μ m pore polycarbonate membrane inserts with or without Matrigel coating (Corning, Shanghai, China), respectively. Briefly, 5000 cells resuspended in serum-free medium were added into the transwell chamber, which was placed in cell culture medium supplemented with 5% fetal bovine serum and was cultured overnight. Cells migrated through the membrane were fixed with 4% paraformaldehyde, followed by staining with GIMSA solution and counting in 3 randomly picked view under microscope.

Flow cytometry

Apoptosis of the SKOV3 and OVCAR-3 cells in in vitro cell culture was evaluated by Annexin V-FITC/PI staining and flow cytometry. Briefly, the cells were collected by trypsin/EDTA (Sigma-Aldrich) treatment, before they were stained using Annexin V-FITC Apoptosis Detection Kit (Solarbio, Beijing, China) following the manufacturer's instructions. The Annexin V-FITC/PI staining of these cells were then evaluated by flow cytometry, and the apoptotic cells were determined by Annexin V-FITC⁺/PI⁺ staining.

Animal studies

All animal maintenance and procedures were carried in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Animal Research Committee of Nanjing Medical University. The tumorigenicity of the SKOV3 cells was evaluated by tumor formation assay using nude mice. Briefly, 10^7 cells resuspended in 0.2 ml of sterile PBS were subcutaneously injected on the back of the nude mice. The tumor volume was measured at day 7, 14, 28 and 35 post injection. The tumor mass was collected for weighting at day 35.

Prediction of lncRNA-miRNA interaction and miRNA-mRNA interaction

Prediction of lncRNA-miRNA and miRNA-mRNA interactions was performed using Encyclopedia of RNA Interactomes (ENCORI) platform (<http://starbase.sysu.edu.cn/>) with default settings.

Luciferase reporter gene assay

To verify the putative miRNA-mRNA and miRNA-lncRNA interaction, the predicted binding sites of miR-455-5p and miR-491-5p on LBX2-AS1 or the 3' untranslated region of E2F2 mRNA were inserted into the 3' untranslated region of Gaussia luciferase gene on the Luciferase reporter vector, which also carries the secreted alkaline phosphatase (SEAP) gene as the internal reference. The reporter vector was co-transfected with miRNA mimic negative control, miR-455-5p mimic or miR-491-5p mimic into the target cells. 24 hours after transfection, the Gaussia luciferase and alkaline phosphatase secreted into the cell culture medium was evaluated using Secreted-Pair Gaussia Luciferase Assay Kit (genecopoeia) following the manufacturer's instructions.

RNA-pulldown assay

To verify the putative miRNA-lncRNA interaction, we used a biotinylated LBX2-AS1 (Ribobio) as probe to enrich LBX2-AS1-interacting miRNAs from the cell lysate of the SKOV3 and OVCAR-3 cells. Briefly, the cells were lysed using a harsh RIPA lysis buffer by pipetting, after which the cell lysate pre-cleared by centrifugation was incubated with the biotinylated LBX2-AS1 for 2 hours at 4 degree with gentle rotation, followed by incubation with streptavidin-conjugated agarose beads (Sigma-Aldrich) for 2 hours under the same condition. miRNAs associated with the beads were then subject to qRT-PCR as described above.

Western blot assay

Protein levels of E2F2 in the SKOV3 and OVCAR-3 cells after various of treatment were evaluated by western blot assay using GAPDH protein as internal reference. The primary antibody for E2F2 (EPR8622, Rabbit monoclonal) and GAPDH (EPR16891, Rabbit monoclonal) as well as the Goat anti-Rabbit secondary antibody (ab205718) were purchased from Abcam (Shanghai, China).

Statistical analysis

Statistical analysis was performed using Graphpad Prism software (Ver. 8.4, GraphPad Software, San Diego, CA, USA). Data were normalized to the mean value of the control group when applicable. Student's t test was used for two-group comparison. One-way analysis of variance using Dunnett's method as the post-hoc test was used for comparison of each experimental group to one control group. One-way analysis of variance using Tukey's method as the post-hoc test was used for multiple group comparison. The p values were marked in histograms and line charts as follows: *, $p < 0.05$, comparing to the control group; **, $p < 0.01$, comparing to the control group; ##, $p < 0.01$, comparing to the LBX2-AS1 knockdown group.

Results

1. Increased expression of LBX2-AS1 in ovarian cancer and its association with patients' reduced survival

In this research, we first explored LBX2-AS1 expression levels in ovarian cancer tissue specimens and non-cancerous ovarian tissue specimens in TCGA ovarian cancer dataset. As shown in Figure 1A, we found that the expression level of LBX2-AS1 in ovarian cancer tissue specimens was significantly higher than that in the non-cancerous ovarian tissue specimens. We next divided the patients' data included in this dataset into LBX2-AS1 high expression group and low expression group based on the LBX2-AS1 expression levels in their ovarian cancer tissue specimens, and we compared the overall survival of these two group of patients. As shown in Figure 1B, the overall survival of patients with LBX2-AS1 expression levels higher than the median value in their ovarian cancer tissue specimens was significantly lower than that of patients with LBX2-AS1 expression levels lower than the median value. We also compared the expression levels of LBX2-AS1 in 60 pairs of patient-derived ovarian cancer tissue specimens and contralateral, non-cancerous ovarian tissue specimens that were obtained during surgery in our facility. As demonstrated in Figure 1C, our qRT-PCR results showed that LBX2-AS1 expression levels in these ovarian

cancer tissue specimens (OC) was significantly higher than that in the contralateral, non-cancerous ovarian tissue specimens (para-cancer). Similarly, we found that LBX2-AS1 expression levels in four commercially purchased human ovarian cancer cell lines—SKOV3, OVCAR-3, Caov-3 and ES-2—were significantly higher than that in one non-malignant human ovarian epithelial cell line—HOSEPICs—as shown in Figure 1D. By separating these 60 ovarian cancer patients according to the LBX2-AS1 expression levels in their ovarian cancer tissue specimens, we found that the overall survival of patients with high LBX2-AS1 expression levels was significantly lower than that of patients with low LBX2-AS1 expression levels in Figure 1E. These results suggested that the expression level of LBX2-AS1 was increased in ovarian cancer, and the high expression of this lncRNA was significantly associated with the reduced overall survival of ovarian cancer patients.

2. LBX2-AS1 knockdown reduced the malignancy of ovarian cancer cells.

Considering that qRT-PCR results presented in Figure 1D have shown that, among the four ovarian cancer cell lines we have examined, LBX2-AS1 expression levels in SKOV3 and OVCAR-3 cells were higher than that in the Caov-3 and ES-2 cells, we chose the SKOV3 and OVCAR-3 cells as models for further LBX2-AS1 knockdown. As shown in Figure 2A, lentiviral transfection of two different LBX2-AS1-targeting shRNAs (sh-LBX2-AS1#1 and #2, respectively) each significantly reduced the expression level of this lncRNA in the two ovarian cancer cell lines in vitro. We then evaluated the malignancy of these ovarian cancer cells with or without LBX2-AS1 knockdown with various cell function assays. We found that, comparing to the negative control cells transfected with non-targeting shRNA (sh-NC), SKOV3 or OVCAR-3 cells with LBX2-AS1 knockdown showed significantly decrease in overall cell growth (Figure 2B), clonogenicity (Figure 2C), trans-well migration and invasion capacity (Figure 2D and 2E, respectively) in vitro as well as tumorigenicity in nude mice (Figure 2G–2H and 2I), while the apoptosis of these two cell lines was significantly increased by LBX2-AS1 knockdown (Figure 2F). These results clearly showed that LBX2-AS1 was integral to the malignancy of ovarian cancer cells.

3. LBX2-AS1 inhibited miR-455-5p and miR-491-5p in ovarian cancer cells via direct interaction

LncRNAs are known to promote cancer progression by interacting with and thus inhibiting the function of cancer suppressive miRNAs [16-17], including LBX2-AS1 [13-14]. We employed ENCORI platform to explore potential LBX2-AS1-interacting miRNAs, and we found miR-455-5p and miR-491-5p as two candidate miRNAs, both of which have been reported as cancer suppressors in ovarian cancer [18-19]. The predicted interaction between LBX2-AS1 and miR-455-5p or miR-491-5p as well as the binding sites were demonstrated in Figure 3A. To verify these predicted interactions, we purchased two different luciferase reporter vectors, on one of which the cDNAs of the two predicted binding sites for miR-455-5p and miR-491-5p on LBX2-AS1 were inserted into the 3' untranslated region of Gaussia luciferase reporter gene (LBX2-AS1-wt), and on the other one of which the cDNA of the two predicted binding sites

were scrambled (LBX2-AS1-mut), and we separately transfected these two different vectors into SKOV3 or OVCAR-3 cells with co-transfection of miR-455-5p mimic, miR-491-5p mimic or miRNA mimic negative control (miR-NC). As demonstrated in Figure 3B and 3C, comparing to that in miR-NC co-transfected cells, co-transfection with miR-455-5p mimic or miR-491-5p mimic significantly reduced the Gaussia luciferase production by the LBX2-AS1-wtreporter vector transfected cells but not the LBX2-AS1-mut reporter vector transfected ones, suggesting that miR-455-5p and miR-491-5p could interact with the predicted binding site on LBX2-AS1 presented in Figure 3A. To further confirm the interaction between LBX2-AS1 and the two miRNAs, we performed RNA pulldown assay in attempt to enrich miR-455-5p and miR-491-5p from ovarian cancer cell lysate using biotinylated LBX2-AS1 as probe. As shown in Figure 3D, we found that miR-455-5p and miR-491-5p could be enriched in the RNA pulldown complex using biotinylated LBX2-AS1 but not a negative control biotinylated RNA constructed by scrambling the sequence of LBX2-AS1, suggesting the direct interaction between LBX2-AS1 and the two miRNAs. To investigate if LBX2-AS1 could functionally inhibit miR-455-4p or miR-491-5p in ovarian cancer cells, we first evaluated the level of these two miRNAs in SKOV3 or OVCAR-3 cells with or without LBX2-AS1 knockdown. As shown in Figure 3E, we found that the levels of these two miRNAs in SKOV3 and OVCAR-3 cells were significantly decreased by LBX2-AS1 knockdown. We further found that the levels of these two miRNAs were significantly lower in the 60 patient-derived ovarian cancer tissue specimens, comparing to that in the paired contralateral healthy ovarian tissue specimen, as shown in Figure 3F. Using the qRT-PCR data in Figure 1C and in Figure 3F, we found that LBX2-AS1 levels in the 60 patient-derived ovarian cancer tissue specimens were negatively associated with that of miR-455-5p or miR-491-5p in Figure 3G. These results suggested that increased LBX2-AS1 level could reduce that of miR-455-5p or miR-491-5p in ovarian cancer cells.

4. 3' UTR of E2F2 mRNA was a direct target of miR-455-5p and miR-491-5p

MiRNAs regulate cancer development often by inhibiting the expression of their target genes through the interaction with their binding sites on the 3' UTR of their target mRNAs^{20,21}. Using ENCORI platform, we found E2F2 as a possible target gene of both miR-455-5p and miR-491-5p, and the predicted interaction between the 3' UTR of E2F2 mRNA and miR-455-5p or miR-491-5p as well as the binding sites were demonstrated in Figure 4A. To verify this novel interaction between E2F2 mRNA and miR-455-5p or miR-491-5p, we constructed luminescence reporter vectors using these two predicted bindings site as described in Figure 3B and 3C. As demonstrated in the right two panel in Figure 4A, co-transfection with miR-455-5p or miR-491-5p mimic could significantly reduce the production of Gaussia luciferase in the two ovarian cancer cell lines governed by the wildtype binding sites (E2F2-wt) but not those governed by the scrambled ones (E2F2-mut), while co-transfection with miRNA mimic negative control (miR-NC) showed minimal effect. These results clearly suggested the interaction between E2F2 mRNA and the two miRNAs. Further analysis showed that low expression of E2F2 had a longer overall survival rate and progression-free survival in OC (<https://kmplot.com/analysis/index.php?p=service&start=1>) (Figure 4B-4C). To further investigate whether miR-455-5p or miR-491-5p could inhibit the expression of E2F2 gene in

ovarian cancer cells, we transfected the SKOV3 and OVCAR-3 cells with miR-455-5p mimic, miR-491-5p mimic or miRNA mimic negative control, after which we compared the protein levels of E2F2 in these cells. As shown in Figure 4D, our western blotting assay results revealed that, comparing to that in the two ovarian cancer cells transfected with miRNA mimic negative control, transfection with miR-455-5p or miR-491-5p mimic significantly decreased the protein level of E2F2 in these cell lines, suggesting that these two miRNAs could directly inhibit the expression of E2F2 gene. Notably, we found that LBX2-AS1 knockdown could also downregulate the protein level of E2F2 in the two ovarian cancer cell lines, which could be abrogated by co-transfection with miR-455-5p or miR-491-5p inhibitor, as suggested by our western blot assay results in Figure 4E, suggesting that LBX2-AS1 could assist the expression of E2F2 gene by inhibiting miR-455-5p or miR-491-5p.

5. LBX2-AS1 increased the malignancy of ovarian cancer cells by regulating the miR-455-5p/miR-491-5p-E2F2 axis

Based on our results presented in Figure 4E, we hypothesized that LBX2-AS1 might participate in the development of ovarian cancer by inhibiting miR-455-5p and miR-491-5p, thus increasing the expression of E2F2 gene. To help verifying this idea, we established E2F2 overexpression in SKOV3 and OVCAR-3 cells by transfection with E2F2 gene overexpressing plasmids, which was confirmed by our western blot assay results presented in Figure 5A. We next achieved LBX2-AS1 knockdown on SKOV3 and OVCAR-3 cells with or without E2F2 overexpression; we found that the downregulation of the overall cell growth of the two ovarian cancer cells by LBX2-AS1 was significantly weakened by the forced expression of E2F2 or the co-transfection with miR-455-5p or miR-491-5p inhibitor, as shown in Figure 5B. We further found that forced expression of E2F2 or the co-transfection with miR-455-5p or miR-491-5p inhibitor significantly abrogated the inhibition of the malignancy of SKOV3 and OVCAR-3 cells by LBX2-AS1 knockdown, as shown in Figure 5C, D, E and F. These results clearly suggested that LBX2-AS1 participates in the cell growth, survival, colony formation, migration and invasion by inhibiting miR-455-5p and miR-491-5p, thus facilitating the expression of E2F2 gene in ovarian cancer cells.

Discussions

LncRNA LBX2-AS1 was first identified in 2016 [22], and the functional research on this lncRNA in cancer biological started very recently. In 2018, two different research groups proposed this lncRNA as a biomarker for poor prognosis in glioma [23-24], after which this lncRNA was found to participate in the development of esophageal squamous cell carcinoma [25], non-small cell lung cancer [26], gastric cancer [14] and hepatocellular carcinoma [13]. Notably, Zhang et al explored LBX2-AS1 expression level in esophageal squamous cell carcinoma and the correlation between LBX2-AS1 expression level and patients overall survival using the data in TCGA esophageal squamous cell carcinoma [12], which inspired us to perform a similar pilot study on ovarian cancer. In this research, we found that, based on the data we obtained from TCGA database and our clinical record, the expression level of LBX2-AS1 was

frequently increased in ovarian cancer, and patients who have high LBX2-AS1 expression levels in their ovarian cancer tissue specimens showed a decreased overall survival, suggesting the cancer promoting role of this lncRNA in ovarian cancer. These results not only suggested the cancer-promoting role of LBX2-AS1 in ovarian cancer development but also present it as a potential biomarker for the prognosis of ovarian cancer patients, which will be further investigated in our future research. We next verified the role of LBX2-AS1 in ovarian cancer cells by several cell functional assays, and the results clearly showed that the expression of this lncRNA is pivotal to the cell growth, survival, colony formation, migration, invasion and tumor formation of ovarian cancer cells, thus further suggesting its cancer-promoting role.

LBX2-AS1 has been found to target microRNA-384 and miR-219a-2-3p in hepatocellular carcinoma and gastric cancer [13-14], respectively, yet the function and mechanism of action of this lncRNA in ovarian cancer as we have revealed haven't been investigated before. Considering that the research on the mechanism of action of LBX2-AS1 in cancer is deficient, in this research we used ENCORI platform to explore possible LBX2-AS1-interacting miRNAs. When selecting miRNA candidates for functional verification, we also took the previous publication records of these candidates into consideration. We noticed that miR-455-5p and miR-491-5p were both reported as cancer-suppressive miRNAs in ovarian cancer [18-19], and in this research, by luminescence reporter gene assay, RNA pulldown assay and cell functional assays, we confirmed that LBX2-AS1 could directly bind to these two miRNAs, by which LBX2-AS1 increased the malignancy of ovarian cancer cells. We further used ENCORI platform to find potential target genes regulated by both miR-455-5p and miR-491-5p, and we found E2F2 as a possible target gene. The cancer-promoting role of E2F2 in ovarian cancer and other types of cancers has been well addressed [27-30]. We therefore hypothesized that LBX2-AS1 might increase the expression level of E2F2 gene in ovarian cancer cells by inhibiting miR-455-5p and miR-491-5p, and this hypothesis was verified by our luminescence reporter gene assay and cell functional assays.

In this research, we found that LBX2-AS1 knockdown could significantly increase the apoptosis of ovarian cancer cells in cell culture, which was counteracted by the inhibition of miR-455-5p or miR-491-5p or by the forced expression of E2F2. Interestingly, Creighton et al reported that E2F2 overexpression could induced p53-dependent apoptosis in p53 competent ovarian cancer cells, while in ovarian cancer cells with disabled p53 signaling, such as SCOV3, effect of E2F2 overexpression on the apoptosis of these cells remained elusive [31]. In this research we also found that the impact of E2F2 overexpression on the apoptosis of SCOV3 and OVCAR-3 cells was indistinctive. It remained to be further investigated how E2F2 participated in the regulation of ovarian cancer cell apoptosis. We also propose that high expression levels of LBX2-AS1 might lead to the increased resistance to chemotherapy or radiotherapy of ovarian cancer cells, considering that Reimer et al have reported the significant association between the high expression levels of E2F2 and increased platinum drug resistance in ovarian cancer patients [32]. These issues will be further addressed in our future research.

A major drawback of this research was the lack of in vivo research. Although we have found that the tumor formation by ovarian cancer cells on nude mice could be delayed by LBX2-AS1 knockdown, these results might not be enough to address that LBX2-AS1 facilitated tumor growth in vivo, considering that

the immune system of the nude mice is impaired. The mechanism of action of this lncRNA as we have proposed based on our cell functional assay results should also be verified in in vivo experiments, which will be performed in our future research.

Conclusions

In summary, we demonstrated that LBX2-AS1 was a novel cancer-promoting lncRNA in ovarian cancer. This lncRNA increased the cell growth, survival, migration, invasion and tumor formation of ovarian cancer cells by inhibiting miR-455-5p and miR-491-5p, thus liberating the expression of E2F2 cancer-promoting gene (Figure 6), which may become a potential molecular marker for ovarian cancer treatment.

Abbreviations

OC: Ovarian Cancer; TCGA: The Cancer Genome Atlas; lncRNAs: long noncoding RNAs; miRNAs: MicroRNAs; E2F2: E2F transcription factor 2.

Declarations

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Availability of data and materials

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

Author contributions

XG and SH designed the study. JC, HW, RT, PX, GL, JM and HW performed the in vitro and animal experiments. JC, HW, RT, PX, GL and HW analyzed the data. JC and HW wrote the paper. All authors read and approved the final paper.

Ethics approval and consent to participate

All animal maintenance and procedures were carried in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Animal Research Committee of Nanjing Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare no Disclosure Statement.

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Figures

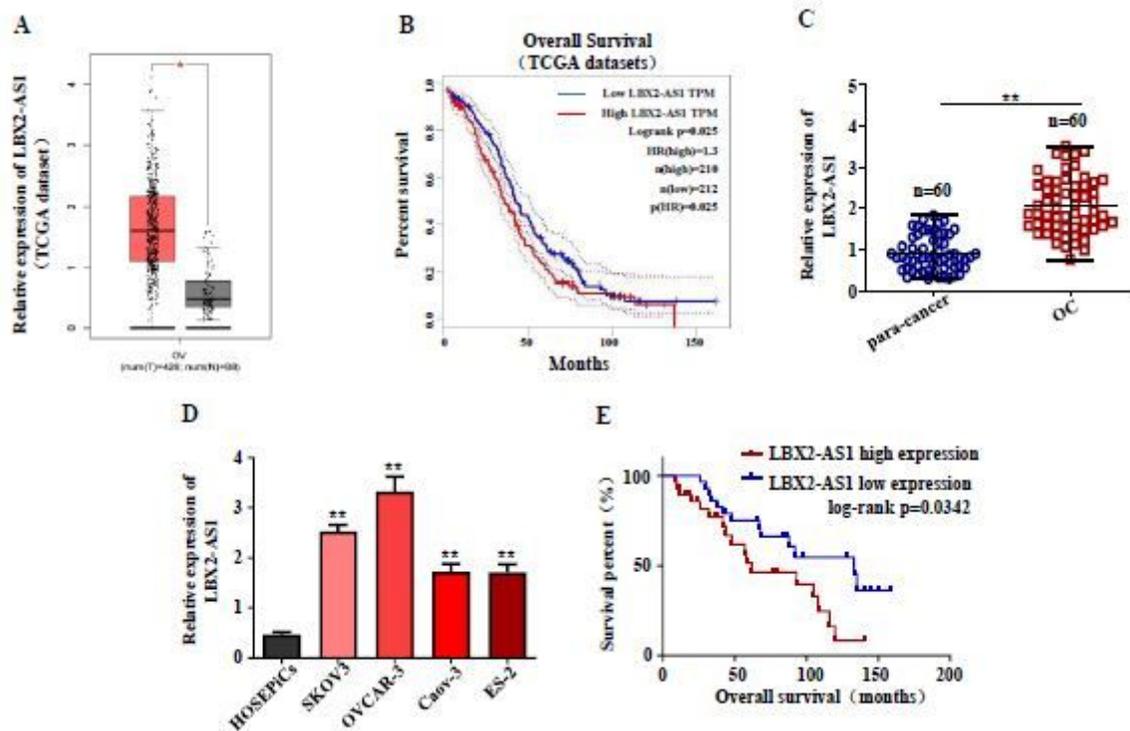


Figure 1

LBX2-AS1 expression was increased in ovarian cancer and its association with patients' reduced survival. (A) expression levels of LBX2-AS1 was found increased in ovarian cancer tissue specimens, comparing to non-malignant ovarian tissue specimens. (B) overall survival of patients with high LBX2-AS1 expression levels in their ovarian cancer tissue specimens were found decreased, comparing to that of patients with low LBX2-AS1 expression levels. (C) expression levels of LBX2-AS1 was found increased in 60 ovarian cancer tissue specimens, comparing to the contralateral, non-malignant ovarian tissue specimens. (D) expression level of LBX2-AS1 in SKOV3, OVCAR-3, Caov-3 or ES-2 cell lines was found higher than that in the HOSEPiCs cells in vitro. (E) among the 60 ovarian cancer patients, overall survival of those with high LBX2-AS1 expression levels in their ovarian cancer tissue specimens were found decreased, comparing to that of patients with low LBX2-AS1 expression levels.

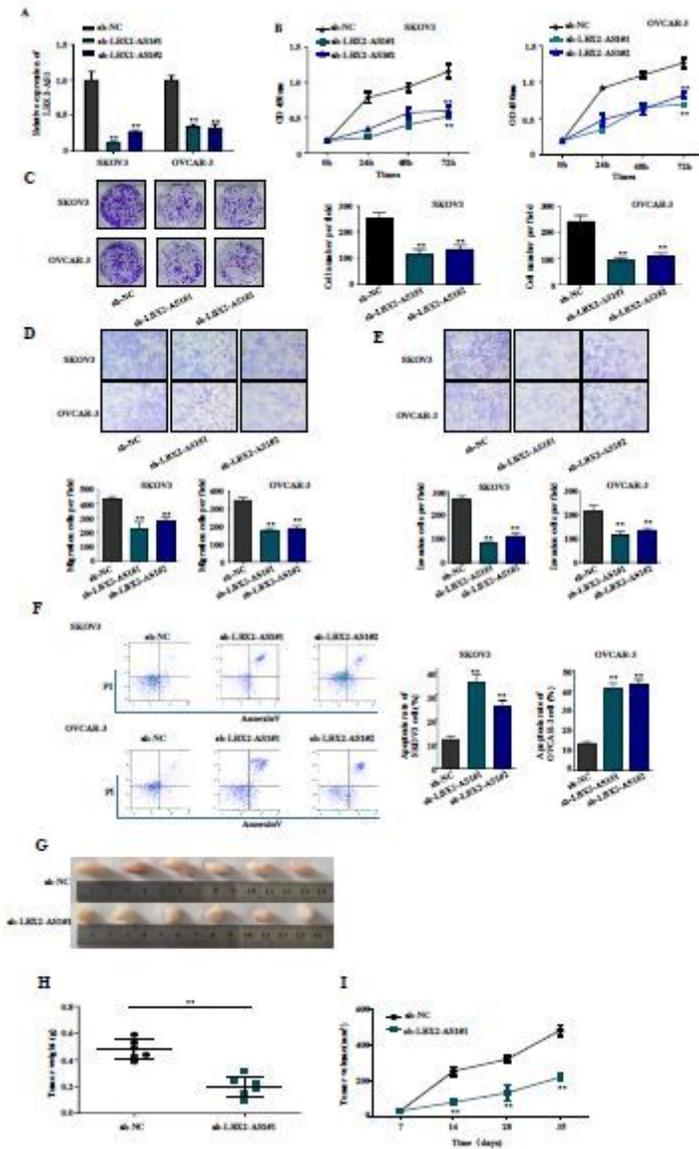


Figure 2

LBX2-AS1 knockdown reduced the malignancy of ovarian cancer cells. (A) LBX2-AS1 expression in SKOV-3 or OVCAR-3 cells was significantly reduced by lentiviral transfection-based knockdown. (B) CCK-8-based cell growth assay results showed that LBX2-AS1 knockdown reduced ovarian cancer cell growth. (C) colony formation assay results showed that LBX2-AS1 knockdown reduced the clonogenicity of SKOV-3 and OVCAR-3 cells. (D) and (E), trans-well migration and invasion assay results showed that LBX2-AS1 knockdown reduced the mobility and invasiveness of SKOV-3 and OVCAR-3 cells. (F) cell apoptosis assay results showed that LBX2-AS1 knockdown increased the apoptosis of SKOV-3 and OVCAR-3 cells in vitro cell culture. Tumor formation assay results showed that LBX2-AS1 knockdown of SKOV-3 cells reduced the tumorigenicity, marked by decreased tumor mass (G-H) and the delayed tumor volume increase (I) at the endpoint.

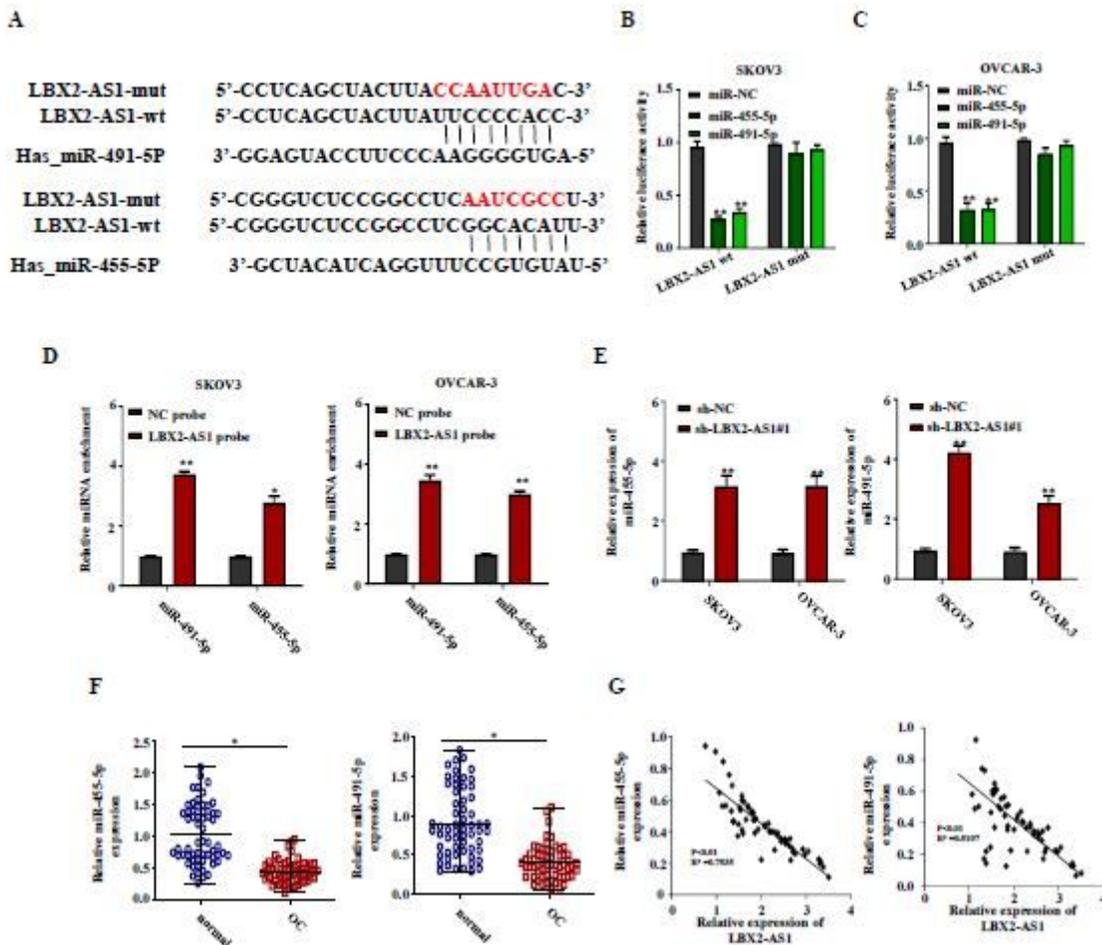


Figure 3

LBX2-AS1 inhibited miR-455-5p and miR-491-5p in ovarian cancer cells via direct interaction. (A) putative interaction between LBX2-AS1 and the two miRNAs were predicted using ENCORI platform. (B-C) luciferase reporter assay results showed that co-transfection with miR-455-5p or miR-491-5p mimic could significantly reduce the production of Gaussia luciferase governed by the putative binding site on LBX2-AS1. (D) RNA pulldown assay results showed that miR-455-5p and miR-491-5p could be enriched from the lysate of SKOV3 and OVCAR-3 cells using biotinylated LBX2-AS1 as bait. (E) qRT-PCR results showed that LBX2-AS1 knockdown could significantly increase the level of miR-455-5p and miR-491-5p in the two ovarian cancer cell lines. (F) expression levels of miR-455-5p and miR-491-5p in the 60 ovarian cancer tissue specimens were significantly lower than that in the contralateral, non-cancerous ovarian tissue specimens. (G) expression levels of LBX2-AS1 were negatively associated with that of miR-455-5p or miR-491-5p in the 60 ovarian cancer tissue specimens.

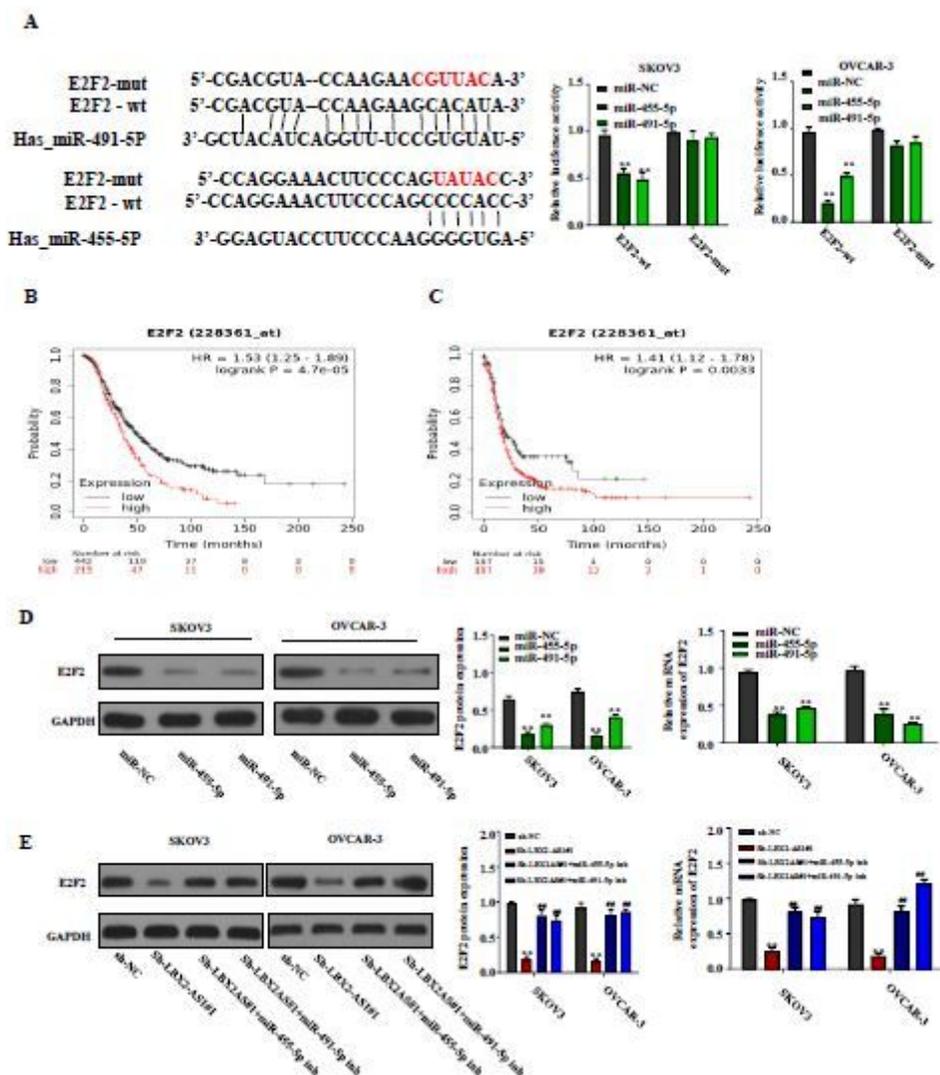


Figure 4

3' UTR of E2F2 mRNA was a direct target of miR-455-5p and miR-491-5p. (A) putative interaction between the 3' UTR of E2F2 mRNA and the two miRNAs were predicted using ENCORI platform (left); luciferase reporter assay results showed that co-transfection with miR-455-5p or miR-491-5p mimic could significantly reduce the production of Gaussia luciferase governed by the putative binding site on the 3' UTR of E2F2 mRNA. (B-C) Kaplan-Meier curve for Kaplan-Meier Plotter datasets of 1657 OC patients for overall survival and 1436 OC patients for progression-free survival. (D) transfection with miR-455-5p or miR-491-5p mimic could significantly reduce the protein level of E2F2 in SKOV3 and OVCAR-3 cells in vitro. (E) downregulation of E2F2 protein level by LBX2-AS1 knockdown was counteracted by transfection with miR-455-5p or miR-491-5p inhibitor in the two ovarian cancer cells.

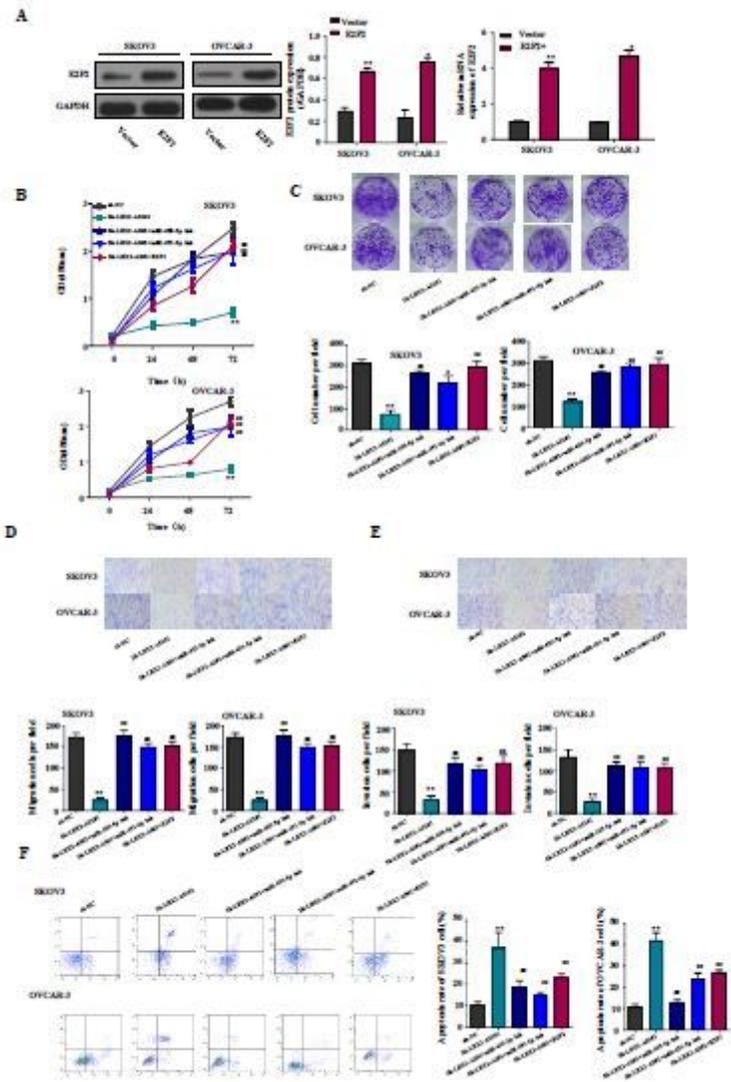


Figure 5

LBX2-AS1 increased the malignancy of ovarian cancer cells by regulating the miR-455-5p/miR-491-5p-E2F2 axis. (A) forced expression of E2F2 gene significantly increased its protein level in SKOV3 and OVCAR-3 cells. (B-E) overall cell growth, colony formation, migration and invasion of the two ovarian cancer cells that were reduced by LBX2-AS1 knockdown were rescued by transfection with miR-455-5p or miR-491-5p inhibitor or the forced expression of E2F2 gene. (F) apoptosis of the two ovarian cancer cell lines provoked by LBX2-AS1 knockdown was rpressed by transfection with miR-455-5p or miR-491-5p inhibitor or the forced expression of E2F2 gene.

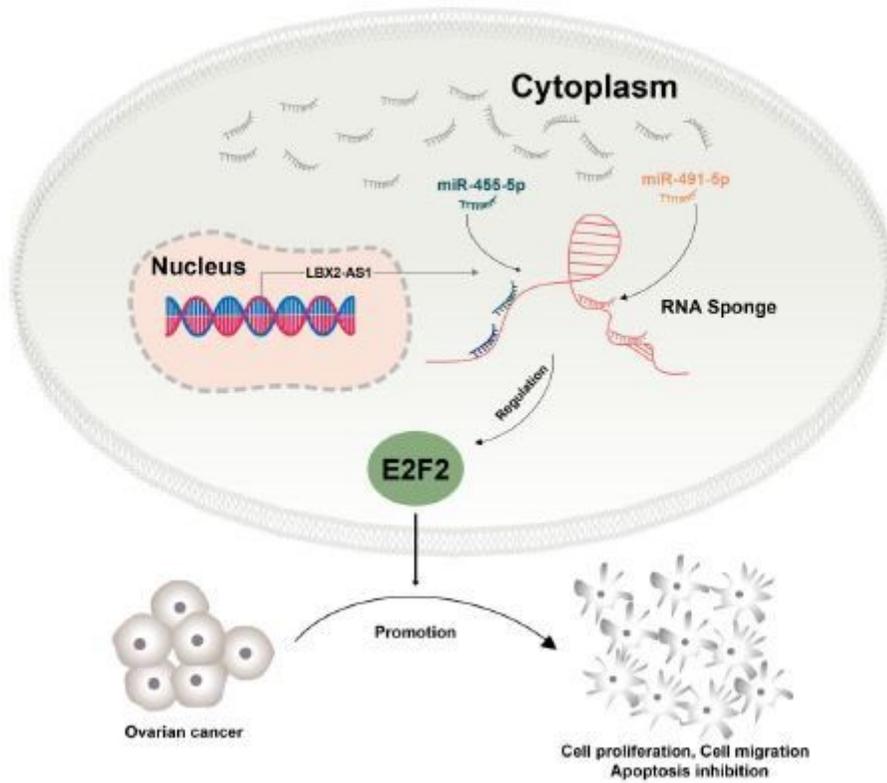


Figure 6

A schematic diagram depicting a potential mechanism by which the LBX2-AS1/miR-455-5p or miR-491-5p/E2F2 axis regulates the proliferation, invasion and apoptosis of OC cells.