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MicroRNA-181b promotes ovarian cancer cell growth and invasion by targeting LATS2

Ying Xia, Yan Gao *

Department of Gynecology, Huadong Hospital, Fudan University, 221 Yan'an West Road, Shanghai, China

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ABSTRACT

MicroRNAs (miRNAs) are strongly implicated in tumorigenesis and metastasis. In this study, we showed significant upregulation of miR-181b in ovarian cancer tissues, compared with the normal ovarian counterparts. Forced expression of miR-181b led to remarkably enhanced proliferation and invasion of ovarian cancer cells while its knockdown induced significant suppression of these cellular events. The tumor suppressor gene, LATS2 (large tumor suppressor 2), was further identified as a novel direct target of miR-181b. Specifically, miR-181b bound directly to the 3′-untranslated region (UTR) of LATS2 and suppressed its expression. Restoration of LATS2 expression partially reversed the oncogenic effects of miR-181b. Our results indicate that miR-181b promotes proliferation and invasion by targeting LATS2 in ovarian cancer cells. These findings support the utility of miR-181b as a potential diagnostic and therapeutic target for ovarian cancer.

1. Introduction

Ovarian cancer is the fourth most common cause of cancer-related mortality among women and considered the most lethal gynecologic malignancy [1]. Despite improvements in combined chemotherapy and biotherapy strategies, the prognosis for survival of patients with ovarian cancer is extremely poor relative to other female reproductive tract malignancies [2,3]. Elucidation of the mechanisms underlying the development and progression of ovarian cancer is therefore critical to develop effective therapeutic options.

MicroRNAs (miRNAs) are small non-coding RNAs ~22 nucleotides in length capable of regulating gene expression at both the transcriptional and translational levels [4]. miRNAs can act as potential oncogenes or tumor suppressor genes owing to their fundamental roles in diverse cellular processes, such as differentiation, proliferation, apoptosis, migration and invasion [5]. However, limited studies have explored their roles in ovarian cancer to date.

LATS2, human large tumor suppressor 2 (also known as KPM), a member of the LATS tumor suppressor family [6], encodes a putative Ser/Thr protein kinase and plays a critical role in mediating Hippo (Hpo) growth inhibitory signaling [7]. Although LATS2 has been established as a tumor suppressor which is downregulated in various cancer types, including lung cancer [8], malignant mesothelioma [9], breast cancer [10] and pancreatic cancer [11], its precise role in ovarian cancer is unclear.

Data from the present study revealed significant upregulation of miR-181b in ovarian cancer tissues, compared to the normal ovarian counterparts. Furthermore, miR-181b promotes ovarian cancer cell growth and invasion by targeting LATS2. Our findings collectively suggest that miR-181b acts as an oncogene and support the utility of both miR-181b and LATS2 as potential biomarkers that may be exploited as targets for ovarian cancer intervention.

2. Materials and methods

2.1. Clinical samples and cell lines

Sixteen human ovarian cancer and six normal ovarian tissues were provided by Huadong Hospital, Fudan University, Shanghai, China. All patients provided consent for use of specimens in research, which was approved by the Ethics Committee of Fudan University. Sample characteristics are described in Supplementary Table S1. Human ovarian cancer cells, SKOV3 (from serous carcinoma) and ES-2 (from clear cell carcinoma), were obtained from Shanghai Institute of Cell Biology, China Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37 °C in 5% CO_2.

* Corresponding author. Fax: +86 21 62483180.
E-mail address: gaoyanhdhos@126.com (Y. Gao).

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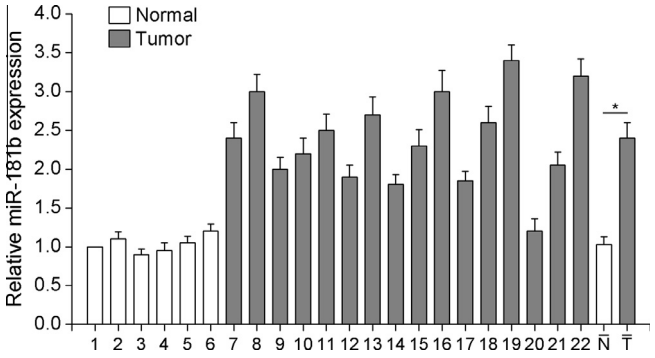

Fig. 1. Expression of miR-181b in human ovarian cancer tissues. qRT-PCR analysis of miR-181b in 16 human ovarian cancer and six normal ovarian tissues. U6 snRNA was used as an endogenous control. $\overline{N}$ and $\overline{T}$ represent average miR-181b expression in normal and tumor tissues, respectively.

2.2. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissues and cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription was performed with Oligo-dT or specific microRNA stem loop RT primers. To determine miR-181b expression levels, qRT-PCR was performed using an EXPRESS SYBR GreenER™ miRNA qRT-PCR kit (Invitrogen) with U6 snRNA as an internal control. For assessment of LATS2 expression, qRT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an internal control. Levels of miR-181b and LATS2 were normalized to those of U6 and GAPDH, respectively, to yield a $2^{-\triangle\triangle Ct}$ value for relative expression of each transcript.

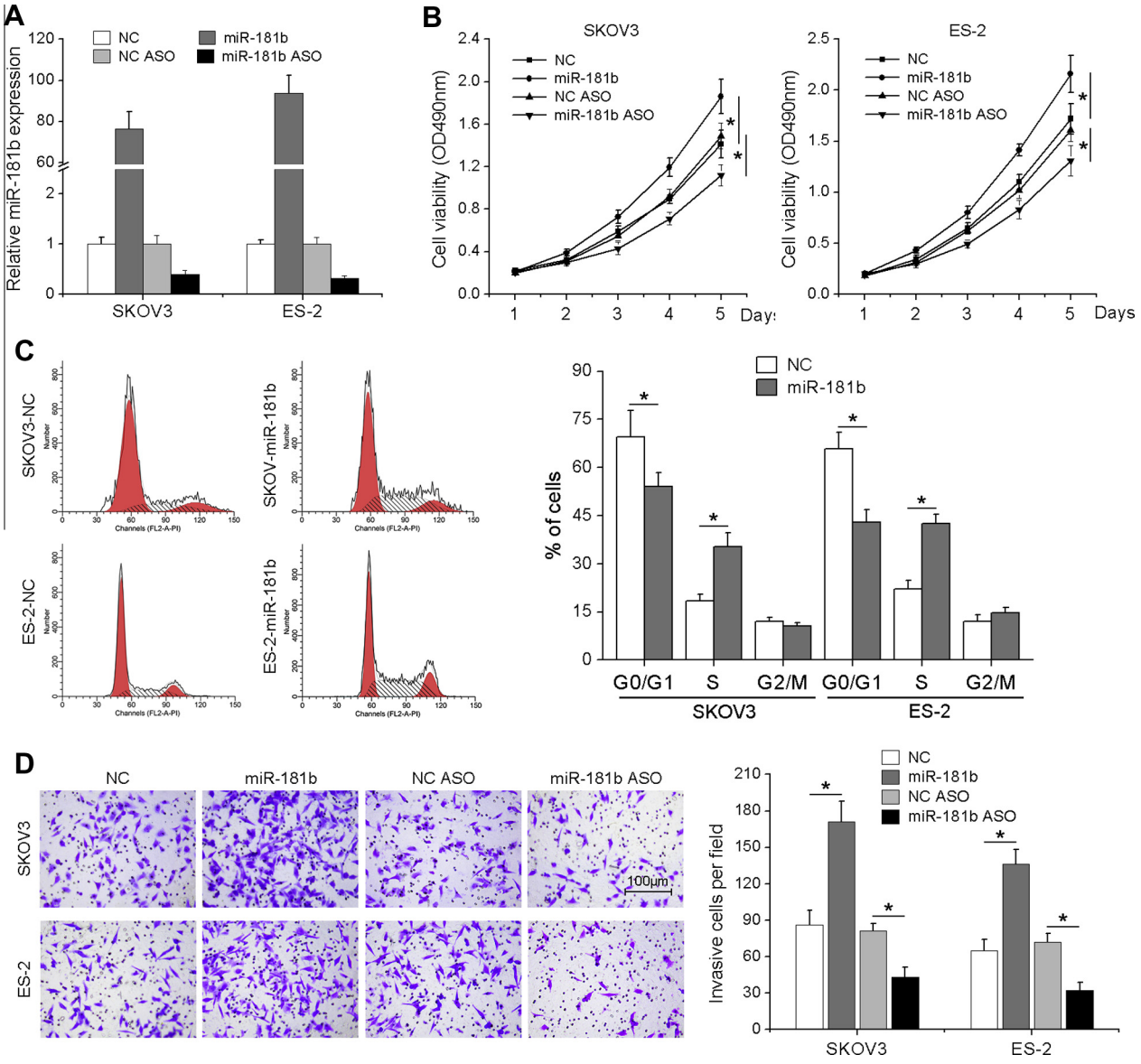

Fig. 2. miR-181b promotes ovarian cancer cell growth and invasion. (A) SKOV3 and ES-2 cells were transfected with miR-181b mimics or miR-181b ASO. miR-181b expression was quantified using qRT-PCR. (B) Determination of cell viability with the MTT assay. (C) Determination of the cell cycle using flow cytometry. (D) Determination of cell invasive ability with the Transwell assay.

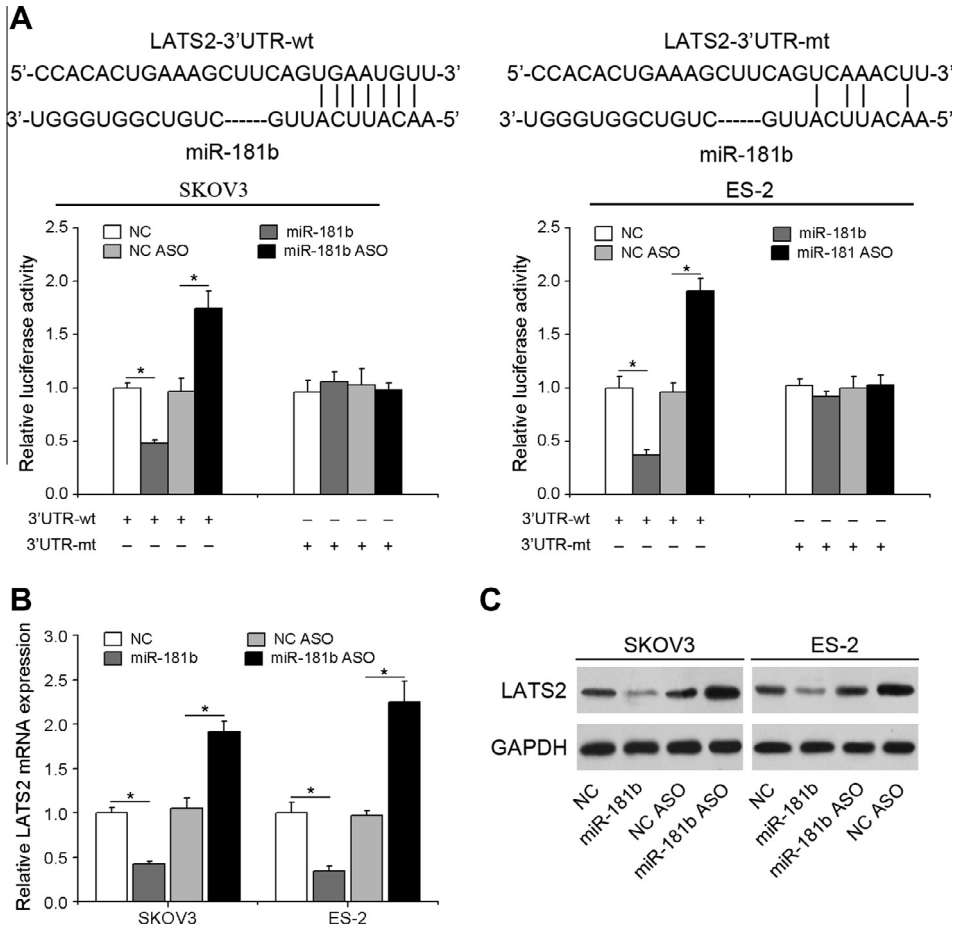

Fig. 3. LATS2 is a direct target of miR-181b. (A) Effect of miR-181b on LATS2-3′UTR-wt and LATS2-3′UTR-mt luciferase reporters. Top, predicted sequences of the miR-181b binding site within the 3′-UTR of LATS2 and mutants containing altered nucleotides in the 3′-UTR region. Bottom, luciferase assays were performed in SKOV3 and ES-2 cells after co-transfection with miR-181b mimics or miR-181b ASO and luciferase reporter plasmid containing either LATS2-3′UTR-wt or LATS2-3′UTR-mt. (B) Analysis of LATS2 mRNA in SKOV3 and ES-2 cells using qRT-PCR with GAPDH as an internal control. (C) Western blot analysis of LATS2 protein expression with GAPDH as an internal control.

2.3. DNA and RNA transfection

SKOV3 and ES-2 cells were transfected with miR-181b mimics, miR-181b antisense oligonucleotide (ASO) or the respective controls (100 nM) (GenePharma, Shanghai, China) using Lipofectamine™ RNAiMAX (Invitrogen) and pcDNA3-LATS2 or pcDNA3 using Lipofectamine 2000 reagent (Invitrogen). Cells were collected for assay at 48 h after transfection.

2.4. Cell proliferation and cell cycle analysis

Transfected cells were seeded into 96-well plates at a density of 2000 cells/well in a final volume of 100 μl, and cultured for 1, 2, 3, 4 and 5 days. MTT (20 μl of 0.5 mg/ml) was added to each well for 4 h at 37 °C, followed by removal of the culture medium and addition of 150 μl DMSO (Sigma, St. Louis, MO, USA). Optical density was measured at a wavelength of 490 nm. For cell cycle analysis, transfected cells were harvested and fixed in 70% ice-cold ethanol at 4 °C for 24 h. Cells were subsequently incubated with 20 μg/ml propidium iodide (Sigma) for 20 min at room temperature, and cell cycle distribution was analyzed using flow cytometry (FACSCalibur, BD Biosciences; San Jose, CA, USA).

2.5. Transwell invasion assay

The cell invasion assay was performed using a Transwell insert chamber coated with Matrigel (BD Biosciences). Cells (1×10^5) suspended in 200 μl RPMI-1640 medium (serum-free) were placed into the upper chamber of the insert with Matrigel. RPMI-1640, with 10% FBS was used as the chemoattractant in the lower chamber. Following incubation of cells for 24 h at 37 °C in a 5% CO_2 humidified incubator, cells located on the top surface of the insert were removed and those on the bottom surface fixed with 4% polyoxymethylene and stained with crystal violet. Cell counting was performed at 200× magnification under an inverted microscope (Olympus, Tokyo, Japan) to determine their relative numbers. The experiment was performed in duplicate and independently repeated at least three times.

2.6. Western blotting

Cells were harvested and lysed with radioimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris–HCl, pH 8.0) containing protease and phosphatase inhibitor mixture (Roche Diagnostics, Indianapolis, IN, USA). Protein concentrations were measured using the BCA protein assay kit (Beyotime, Haimen, China). Lysates were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane (Merck Millipore, Billerica, MA, USA). Next, membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature and probed with primary antibodies against LATS2 and GAPDH overnight. After incubation with horseradish peroxidase-conjugated secondary

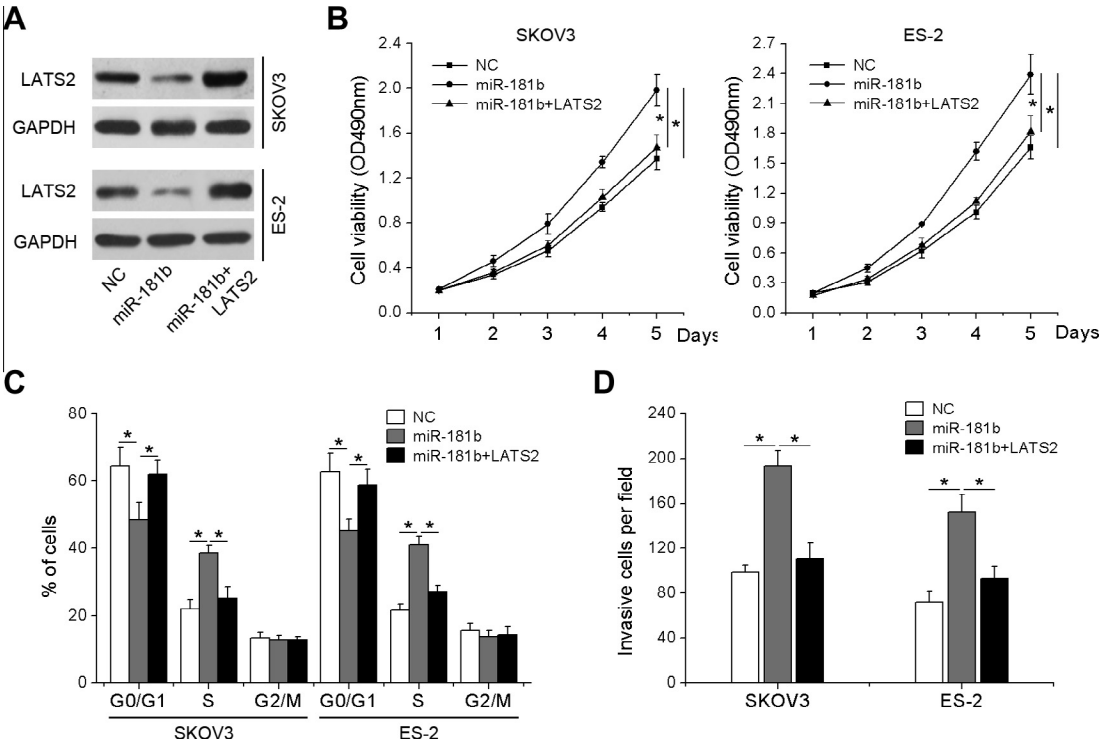

Fig. 4. LATS2 is involved in miR-181b regulation of ovarian cancer cell growth and invasion. SKOV-3 and ES-2 cells were transfected with miR-181b or co-transfected with miR-181b and pcDNA3/LATS2. (A) Western blotting analysis of LATS2 expression with GAPDH as an internal control. (B) Determination of cell viability with the MTT assay. (C) Flow cytometry analysis of the cell cycle. (D) Determination of cell invasion ability with the Transwell assay.

antibodies, bound proteins were detected with Immobilon chemiluminescent substrate (MerckMillipore).

2.7. Plasmid construction and the luciferase reporter assay

The sequence in the 3′-UTR region of human LATS2 targeted by miR-181b was predicted with Targetscan (www.targetscan.org) and amplified from genomic DNA of normal cells. LATS2 3′-UTR and a sequence with mutation of three nucleotides in the miR-181b target site were synthesized and cloned into pGL3 promoter vector to generate the recombinant vectors, pGL3-LATS2-3′UTR-wt and pGL3-LATS2-3′UTR-mt, respectively. For the luciferase reporter assay, ovarian cancer cells were co-transfected with pGL3-LATS2-3′UTR-wt or pGL3-LATS2-3′UTR-mt and miR-181b mimic or miR-181b ASO. At 48 h after transfection, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to that of *Renilla* luciferase.

2.8. Statistical analysis

Data were expressed as mean values ± SD from at least three separate experiments. Student's *t*-test and one-way analysis of variance (ANOVA) were used to compare mean values from different samples. $^{*}P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-181b is upregulated in human ovarian cancer tissues

To assess miR-181b expression in ovarian cancer, 16 human ovarian cancer and six normal ovarian tissues were subjected to qRT-PCR. As shown in Fig. 1, miR-181b expression was elevated in almost all ovarian cancer tissues, compared to the normal ovarian counterparts, indicating a role of miR-181b in ovarian cancer progression.

3.2. miR-181b promotes ovarian cancer cell proliferation and invasion

To establish whether miR-181b plays a role in tumorigenesis, the ovarian cancer cell lines, SKOV3 and ES-2, were transfected with miR-181b mimics or miR-181b ASO, and miR-181b expression was verified using qRT-PCR (Fig. 2A). The MTT assay revealed that miR-181b overexpression induced a significant increase in the growth rate of SKOV3 and ES-2 cells, compared to the corresponding controls (Fig. 2B). Conversely, SKOV3 and ES-2 cells transfected with miR-181b ASO showed a significantly decreased cell growth rate. These results suggest that miR-181b exerts a growth-promoting function in ovarian cancer cells. We subsequently investigated whether miR-181b has an impact on cell cycle progression of ovarian cancer cells. In cell lines transfected with miR-181b mimics, the percentage of cells in the G1/G0 phase was significantly decreased and that in the S phase significantly increased, as shown in Fig. 2C. Thus, the growth-promoting function of miR-181b may be due to enhancement of cell cycle progression at G1/S transition in ovarian cancer cells.

The effect of miR-181b on cell invasion was determined using the Transwell assay with Matrigel. As shown in Fig. 2D, transfection of miR-181b mimics led to a significant increase in the invasive ability of both SKOV3 and ES-2 cells while miR-181b ASO induced a decrease in cell invasion. These results clearly suggest that miR-181b enhances ovarian cancer cell invasion.

3.3. LATS2 is a direct target of miR-181b

To clarify the mechanism underlying miR-181b activity in ovarian cancer cell growth and invasion, we performed a bioinformatics search for potential targets of miR-181b. Among the

targets identified, LATS2 was selected for further analysis. Two luciferase reporters, one containing wild-type LATS2 3′-UTR with the miR-181b binding site (LATS2-3′UTR-wt) and another containing mutant LATS2 3′-UTR (LATS2-3′UTR-mut), were constructed with the aim of determining whether LATS2 is a direct target of miR-181b. In the luciferase reporter assay, miR-181b suppressed >50% transcriptional activity of the LATS2-3′UTR-wt reporter, compared with control, but did not affect the activity of the mutant reporter gene (Fig. 3A). Conversely, inhibition of miR-181b caused a marked increase in luciferase reporter activity under control of the 3′-UTR of LATS2. qRT-PCR and Western blot analyses further demonstrated that miR-181b overexpression induces a significant decrease in LATS2 mRNA and protein expression in SKOV-3 and ES-2 cells while silencing of miR-181b enhances LATS2 expression in these cell lines. Our results collectively indicate that miR-181b directly suppresses LATS2 mRNA and protein levels.

3.4. LATS2 is involved in miR-181b-induced ovarian cancer cell growth and invasion

To further establish whether miR-181b-induced ovarian cancer cell growth and invasion are mediated by repression of LATS2, LATS2 lacking 3′-UTR was ectopically expressed together with miR-181b in SKOV-3 and ES-2 cells (Fig. 4A). As shown in Fig. 4B, reintroduction of LATS2 significantly suppressed the proliferation of miR-181b-overexpressing ovarian cancer cells. In addition, enforced expression of LATS2 clearly counteracted the enhancement of cell cycle progression at G1/S transition induced by miR-181b (Fig. 4C). Restoration of LATS2 expression led to a marked decrease in the invasive activity of miR-181b-expressing ovarian cancer cells. The finding that induction of LATS2 expression abrogates miR-181b-induced cell growth and invasion suggests that LATS2 is a functional mediator of miR-181b in ovarian cancer progression.

4. Discussion

Ovarian cancer is the most lethal gynecological malignancy identified to date [1]. The American Cancer Society estimates over 20,000 new cases of ovarian cancer and about 15,500 deaths owing to the disease every year [12]. Thus, strategies are urgently needed for the effective diagnosis, treatment and prognosis of ovarian cancer. Recent studies have revealed that miRNAs play key roles in cancer development and potentially serve as biomarkers for prediction and prognosis in various cancers [13,14]. Here, we aimed to clarify the biological role of miR-181b in human ovarian cancer. Our experiments showed significant elevation of miR-181b in ovarian cancer tissues, compared to normal ovarian tissues. Overexpression of miR-181b promoted the growth and invasion of ovarian cancer cells, accompanied by an increase in the number of cells in the S phase. Moreover, miR-181b suppressed LATS2 expression by directly targeting its 3′-UTR region.

miR-181b belongs to the miR-181 family, which comprises four members: miR-181a, miR-181b, miR-181c, and miR-181d [15]. Recent reports have provided evidence that members of the miR-181 family function as either onco-microRNAs or tumor suppressors [15–18]. For instance, miR-181a plays a positive role in ovarian cancer progression and promotes TGF-β-mediated epithelial-to-mesenchymal transition [16]. Conversely, miR-181c inhibits neuroblastoma cell growth and metastasis-related traits [17] while miR-181d suppresses proliferation and triggers cell cycle arrest and apoptosis in glioma cell lines [18].

miR-181b exhibits oncogenic properties and is upregulated in several cancers, including colorectal cancer, breast cancer, hepatocellular carcinoma, cervical cancer, and prostate cancer [19–23]. However, miR-181b has been shown to be a potential tumor suppressor in glioma [24,25]. In other studies, ectopic expression of miR-181b promoted growth, clonogenic survival, migration and invasion of hepatocellular carcinoma and prostate cancer cells [21,23]. The current study has shown that miR-181b is upregulated and promotes human ovarian cancer cell growth and invasion. Therefore, our results support the view that miR-181b mainly functions as an oncogenic miRNA in ovarian cancer.

Since miRNAs usually exert their functions by suppressing the expression of target mRNAs, we further aimed to identify miR-181b target genes in ovarian cancer. LATS2 was identified as a critical downstream target using different prediction algorithms. Luciferase reporter assays showed that miR-181b triggers a significant decrease in the luciferase activity of wild-type LATS2 3′-UTR reporter but does not affect mutant reporter activity. Furthermore, transfection of miR-181b mimics suppressed LATS2 expression in ovarian cancer cells. In view of these results, we propose that miR-181b directly downregulates LATS2 gene expression via binding to its 3′-UTR region.

LATS2, located in human chromosome 13q11-12, is a member of the LATS tumor suppressor family [6]. Loss of heterozygosity at the LATS2 locus is reported in ovarian and breast cancer [26,27]. Recent studies have shown that LATS2 regulates diverse cellular processes, such as proliferation, apoptosis, angiogenesis, migration and invasion [8–11,28–30]. For example, silencing of LATS2 expression promotes cell survival, tube formation and invasion in breast cancer, while ectopic expression of LATS2 decreases cell survival and invasion [31]. LATS2 also acts as a negative regulator of cell growth by controlling G1/S and/or G2/M transition and inducing apoptosis [8,9,11,28]. Here, we showed for the first time that miR-181b-induced cell growth and invasion is mediated, in part, by suppressing LATS2.

In summary, our study provides novel evidence that miR-181b promotes the growth and invasion of ovarian cancer cells by targeting LATS2. Therefore, miR-181b and LATS2 may represent potential predictive and therapeutic targets for ovarian cancer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.027.

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