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Long non-coding RNA ASB16-AS1 enhances cell proliferation, migration and invasion via functioning as a ceRNA through miR-1305/Wnt/β-catenin axis in cervical cancer

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ABSTRACT

Background: Cervical cancer (CC) is one of the most common cancers in women. Long non-coding RNAs (lncRNAs) have been proposed as therapeutic targets in CC. Hence, the present study evaluated the effect of ASB16-AS1 on CC via regulating miR-1305.

Methods: Differentially expressed lncRNAs associated with CC were screened using bioinformatics database. The expression of ASB16-AS1 and miR-1305 were measured by qRT-PCR in CC tissues and CC cells. Cell proliferation was assessed by CCK-8 and colon formation assays. Cell abilities of migration and invasion were detected by Transwell migration and invasion assays. Luciferase report assays were used to explore the correction between ASB16-AS1, miR-1305 and Wnt2 in CC. Western blot assay detect the activity of Wnt/β-catenin pathway. The xenograft tumor in nude mice was observed to evaluate tumor formation *in vivo*.

Results: In our study, we showed that the expression of ASB16-AS1 was increased while miR-1305 reduced was re in CC. Clinically, ASB16-AS1 and miR-1305 were correlated with poor-associated clinicopathological features of CC patients. Knockdown of ASB16-AS1 reduced CC cells proliferation, migration and invasion abilities by regulating miR-1305 *in vitro* and *in vivo*. Moreover, miR-1305 was directly bound to ASB16-AS1 and Wnt2, regulated their expression negatively. Western blot assays showed that ASB16-AS1 functioned as an oncogene by Wnt/β-catenin pathway.

Conclusions: This study reveals that ASB16-AS1 promotes cell proliferation, migration, invasion via binding miR-1305 with Wnt2, and enhancing the Wnt/β-catenin pathway. ASB16-AS1 may play a new therapeutic target for CC.

1. Introduction

Cervical cancer (CC) is the fourth most common leading cause of cancer-related deaths in women. There are 569,847 new cases world-wide, and 311,365 deaths in 2018 [1]. Despite the promotion of human papillomaviruses (HPV) vaccine, incidence rate of CC is still the first in gynecological tumors, especially in developing countries [2]. Although surgical treatment and radiotherapy are effective treatment for early CC patients, the prognosis of patients with advanced stages CC remains poor [3,4]. Recently, molecular targeted therapies significantly improve the outcome of various kinds of cancers, including breast cancer, lung cancer, colon cancer and so on [5–7]. But most patients with metastatic CC are not sensitive to currently molecular targeted therapies. Consequently, further researches on molecular mechanisms of progression and metastasis of CC, are conducive to finding more effective treatments for CC.

Long non-coding RNAs (lncRNAs) are a kind of non-coding RNAs that transcripts longer than 200 nucleotides, with no protein-coding function [8,9]. LncRNAs play important roles in many processes of cellular functions, for instance, X chromosome imprinting, chromatin

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modification, and immune response. Meanwhile, lncRNAs are involved in dysregulated in human cancers [10,11]. LncRNA TPT1-AS1 promotes CC cell growth and metastasis by targeting miR-324 [12]. TDRG1 functions as a competing endogenous lncRNA (ceRNA) to regulate MAPK1 via competitive sharing miR-326 in CC [13].

LncRNA ASB16-AS1 is located on 17q21, and the length is 2275 bp. Zhang et al. has reported ASB16-AS1 could function as an oncogenic regulator in glioma cells. In current study, we first identified ASB16-AS1 was up-regulated in CC tissues, moreover, we also confirmed ASB16-AS1 promoted CC tumorigenesis and metastasis, via acting as a ceRNA for miR-1305, thereby preventing its association with target Wnt2 *in vitro* and *in vivo*. Our results provide that ASB16-AS1 exerts a critical role in CC progression and may be useful candidate for CC diagnosis and therapy.

2. Materials and methods

2.1. Ethics statement

The protocol of this study was approved by the ethics committee of the Second Affiliated Hospital of Harbin Medical University (China; approval no. KY: 2018-334).

2.2. Clinical samples and cell culture

CC tissues and normal cervical tissues were obtained from patients who underwent surgery between November 2015 and November 2018 at the Second Affiliated Hospital of Harbin Medical University. Patients diagnosed with CC and with other non-cancerous gynecological diseases were included in our study. Only those patients with no radiation or chemotherapy were included in the study. All patients signed informed consents and agreed to use surgical specimens for this clinic research.

The CC cell lines HeLa, Ca-ski, HT-3, and SiHa together with normal cervical cells were from American Type Culture Collection (ATCC, USA). Cells were cultured in DEME medium (Gibco, USA) containing 10 % fetal bovine serum (FBS, Gibco, USA), and 100 μg/L penicillin/streptomycin. Cells grew in a cell incubator (Thermo Fisher Scientific Inc., USA) in 5 % CO_2 at 37 ˚C. The cells used were no more than 20 passages.

2.3. Cell transfection

SiRNA against ASB16-AS1 (si-ASB16-AS1), siRNA against negative control (si-NC), hsa-miR-1305 inhibitor (miR-1305 inhibitor), inhibitor NC (inhibitor-NC), were synthesised and purchased from GenePharma (Shanghai, China). ASB16-AS1 siRNA-1 sequence was 5′-GGTTCTGAA TCATTCAGTT-3′ and ASB16-AS1 siRNA-2 sequence was 5′-AAGCATC TTCAGTTTTCATATGA-3′.

HeLa cells and SiHa cells were seeded in 12-well plates overnight before transfection. Cell transfection was performed when cell fusion reached a confluence of 50 %–60 %. lipofectamine™ 2000 (Invitrogen, USA) was mixed with siRNA or inhibitor, according to the manufacturer's protocol. After transfection 48 h–72 h, cells were collected for subsequent experiments.

2.4. RNA extraction and quantitative RT-PCR

Total RNA was extracted from the specimens and cells using TRIzol (Invitrogen, USA). The RNA was dissolved in diethyl pyrocarbonate (DEPC) ultrapure water and the absorbance was measured at 260 /280 nm using a spectrophotometer (Beijing UESIPHY Development Co., Ltd., China). The quality and concentration of total RNA were assessed and determined. PrimeScript RT Master Mix (TOYOBO, Japan) was used to reverse transcribed RNA to cDNA. qRT-PCR analyses were performed with SYBR Green Mix (TOYOBO, Japan). U6 was used as the internal reference for miRNAs and GAPDH as the internal reference for the other genes. The ratio of the expression of target gene in the experimental group to that in the control group was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primers used for reverse transcription and PCR in this study.

Gene	Primer sequence	
ASB16-16	Forward	5′-CGGCCCTGAGGCAAACATAC-3
	Reverse	5′-TGAAACACTGCGCCAACTTC-3′.
GAPDH	Forward	5′-GCCTGCTTCACCACCTTCT-3′
	Reverse	5′-GAACGGGAAGCTCACTGG-3′
Wnt2	Forward	5′-CCGAGGTCAACTCTTCATGGT-3′
	Reverse	5′-CCTGGCACATTATCGCACAT-3′
miR-1305	Forward	5′-ACAGGCCGGGACAAGTGCAATA-3′
	Reverse	5′-GCTGTCAACGATACGCTACGTAACG-3′
U6	Forward	5′-ATTGGAACGATACAGAGAAGATT-3′
	Reverse	5′-AGGAACGCTTCACGAATTTG-3′

The primers of ASB16-AS1, miR-1305 were designed and purchased from Ribobio (Guangzhou, China) (Table 1).

2.5. CCK-8 assay

Cell Counting Kit-8 (CCK-8) (Solarbio, China) was used to detect cell proliferation ability. After 6 h of cells transfection, 1×10^3 cells per well were planted in 96-well plates. After the cells were cultured for 24 h,48 h,72 h, and 96 h, each well was added by 10 μl of CCK8, and incubated at 37 ˚C for 2 h. The cell viability was calculated at a wavelength of 450 nm.

2.6. Colony formation assay

Transfected CC cells were added to 6-well plates, and incubated for 14 days, then fixed in 4 % paraformaldehyde and stained with 0.1 % crystal violet (Solarbio, China). Colony of a diameter > 40 mm are imaged and counted.

2.7. Transwell migration and invasion assay

Transwell chambers (Corning, USA) with Matrigel (Becton, USA) were used to assess cell invasion ability, while the transwell chambers without Matrigel were used to assess cell migration. Matrigel melted into liquid at 4 ˚C overnight, and then mixed with DEME at a ratio of 1:8. Then 40 μl diluted Matrigel was added to each chamber carefully and incubated at 37 ˚C to make Matrigel polymerized into gel. After transfection 24 h, $1-2 \times 10^4$cells(depending on different experiments, consistent between groups) with free-FBS DMEM, were seeded in each upper chamber, the lower chambers were added with DEME containing 10 % FBS simultaneously.

After incubated for 48 h, the cells fixed with 4 % paraformaldehyde, stained with hematoxylin and eosin (Solarbio, China). Five visual fields were randomly selected under an inverted microscope (OLYMPUS, Japan).

2.8. Luciferase assay

2×10^4 cells per well were seeded in 24-well plates in a cell incubator in 5 % CO2 at 37 ˚C overnight. miR-1305 inhibitor or NC inhibitor were transfected, according to the cell transfection part description. The activities of Luciferase and Renilla were assessed after treated by luciferase reporter assay kit (Promega, USA) for 48 h, according to the manufacturer's protocol.

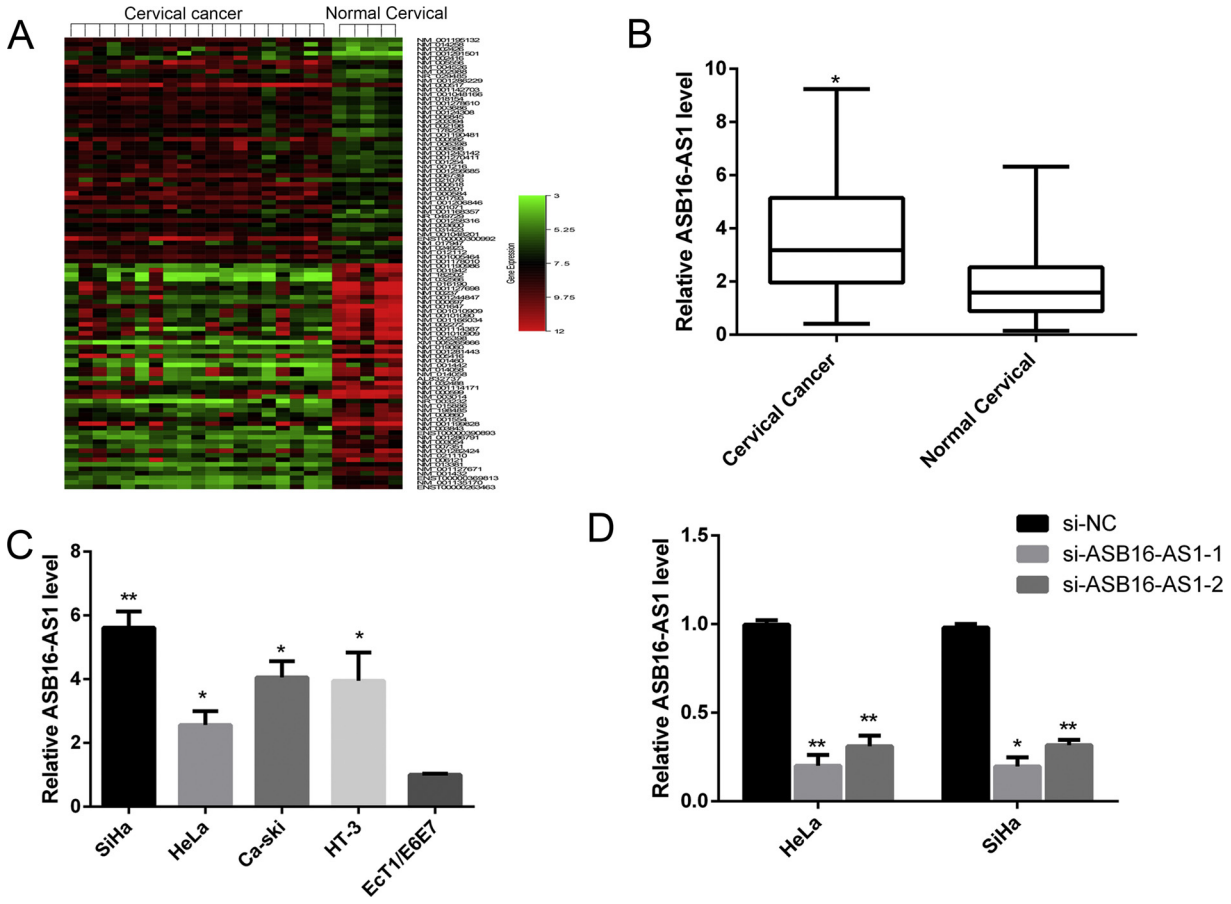

Fig. 1. ASB16-AS1 is upregulated in CC tissues and cell lines. **(a)** Heat map with hierarchical clustering of the top differentially expressed lncRNAs between CC samples and normal samples (> 2-fold; $P < 0.05$). **(b)** qRT-PCR was used to detect ASB16-AS1 expression in CC samples and normal samples(n = 60). **(c)** qRT-PCR was used to examine the expression of ASB16-AS1 in CC cell lines and non- carcinoma cervical epithelial cell line. **(d)** qRT-PCR was used to examine the expression of ASB16-AS1 in HeLa and SiHa cells after transfection with si-ASB16-AS1-1 or si-ASB16-AS1-2 or si-NC. Date are shown as mean ± SD from three independent experiments, by Student's test. *P < 0.05;**P < 0.01.

Table 2
Correlation between the expression of ASB16-AS1 or miR-1305 and clinicopathological characteristics in 60 cases of cervical cancer. (n = 60).

Clinical parameters	Expression level		*P value*	Expression level		*P* value
	ASB16-AS1^{High}	ASB16-AS1^{Low}		miR-1305^{High}	miR-1305^{low}	
Age(year)						
≥50	11	14	0.432	15	10	0.19
<50	19	16		15	20	
FIGO stage						
I	13	24	0.003**	23	14	0.017*
II	17	6		7	16	
Tumor Size(cm)						
<4cm	11	26	< 0.001**	26	11	< 0.001**
≥4cm	19	4		4	19	
Lymph node metastasis						
No	12	21	0.020*	22	11	0.004**
Yes	18	9		8	19	
Histology grade						
Well and moderate	6	16	0.007**	15	7	0.032*
Poor	24	14		15	23	
Histology						
Squamous	23	21	0.559	25	19	0.08
Adenocarcinoma	7	9		5	11	

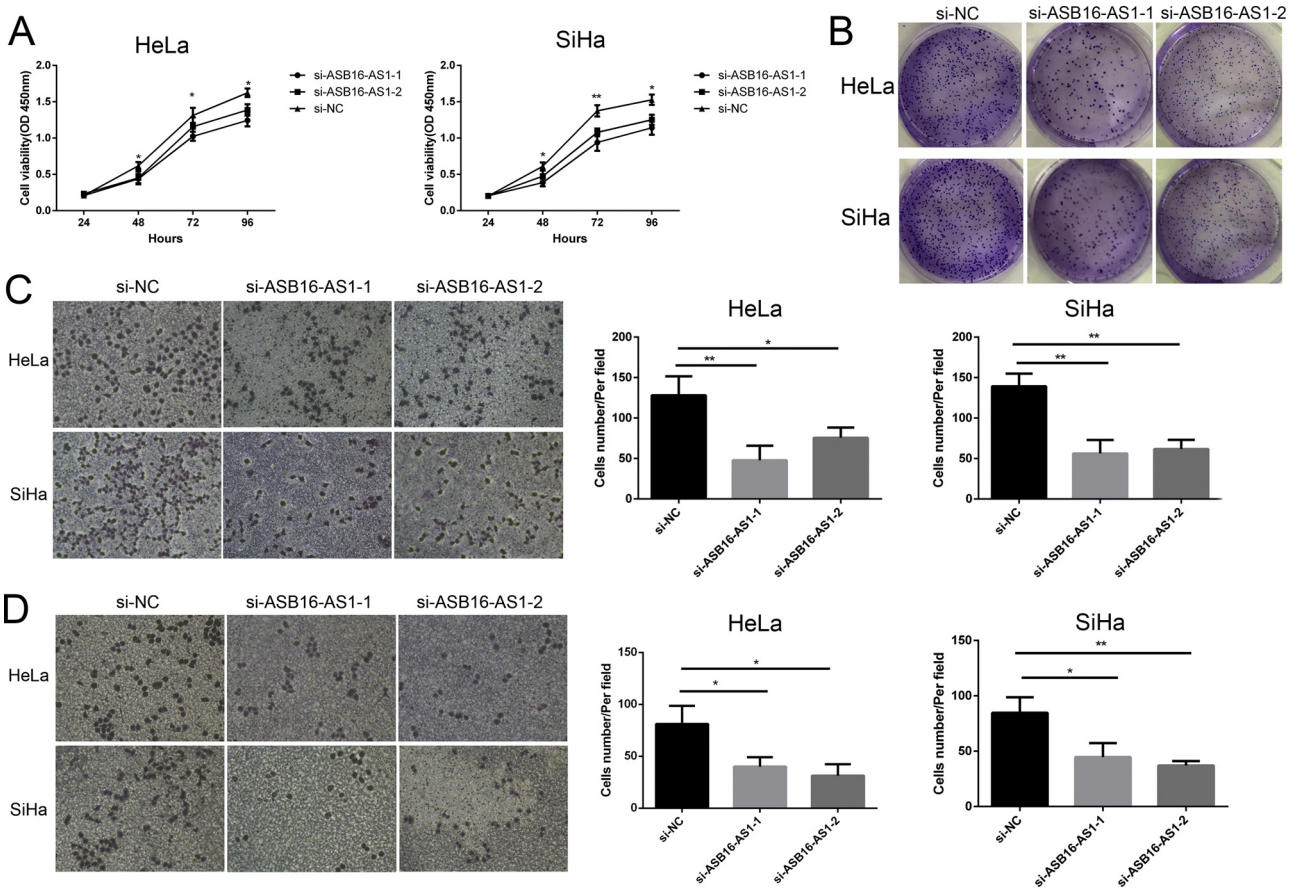

Fig. 2. ASB16-AS1 promotes cell proliferation, migration and invasion in CC cells. **(a)** CCK8 assays were used to determine the viability of si-ASB16-AS1-1 or si-ASB16-AS2 transfected CC cells. **(b)** Colony formation assays were performed to determine the proliferation of si-ASB16-AS1-1 or si-ASB16-AS2 transfected CC cells. The migration **(c)** and invasion **(d)** abilities of HeLa and SiHa cells were detected in transwell assays (without or with Matrigel) after transfection with si-ASB16-AS1-1 or si-ASB16-AS1-2 or si-NC. Date are shown as mean ± SD from three independent experiments, by Student's test. *P < 0.05;**P < 0.01.

2.9. Western blot assay

After transfection for 72 h, cells were collected to use in Western blot. Western blot analysis was performed using standard protocols. The following primary antibodies were used: Wnt2(ab27794, Abcam, USA), β-catenin(ab32572, Abcam, USA), c-myc(ab185656, Abcam, USA), p21(ab218311, Abcam, USA),and β-actin (TA-09, ZSGB, China). Secondary antibodies were used: anti-Mouse Ig DyLight™ 800(18-4516-32, Rockland, USA) and anti-Rabbit IgG DyLight™ 800(18-4517-32, Rockland, USA). Secondary antibodies were chosen by different primary antibodies. Protein expression levels were detected by Odyssey system (LI−COR, USA). β-actin was used as an internal protein reference and the ratio of the gray value of the target band to the internal reference band was used as the relative expression level of the protein.

2.10. In vivo experiments

The 4 weeks BALB/c female nude mice were purchased from Institute of Laboratory Animals in Chinese Academy of Medical Sciences (Shanghai, China). 1×10^7 stably transfection cells were injected into the back of each mice. All the nude mice were fed under SPF condition. Following the appearance of the tumor, the maximum diameter (L) and the minimum diameter (W) of the tumor were measured with a vernier caliper, and the tumor volume(V) was calculated according to the formula: V (mm^3) = (L × W^2)/2. The tumor size and weight of nude mice were measured every 5 days. The nude mice were sacrificed 35 days later.

2.11. Statistical analysis

SPSS 21.0 was employed for statistical analysis. The measurement data were expressed as mean ± standard deviation. The data comparison among multiple groups was performed by one-way analysis of variance (ANOVA), with Tukey's post hoc test. Data comparison at different time points was performed by repeated measures ANOVA, with Bonferroni post hoc test. The difference was statistically significant at $p < 0.05$.

3. Results

3.1. ASB16-AS1 is up-regulated in CC tissues and cell lines

Based on the data analysis for GSE39001 microarray chip, we found that ASB16-AS1 was highly expressed in CC tissues in comparison to normal cervical tissues (fold change > 2; $P < 0.05$; Fig. 1A). Based on these results, qRT-PCR was performed to explore lncRNA ASB16-AS1 expression in CC tissues. Results showed the expression of ASB16-AS1 was significantly up-regulated in CC tissues compared to normal cervical tissues samples ($P < 0.05$; Fig. 1B). Moreover, as shown in Table2, the statistical results indicated that overexpression of ASB16-AS1 was dramatically associated with FIGO stage ($P = 0.003$), tumor size ($P < 0.001$), lymph node metastasis($P < 0.02$) and histology grade($P = 0.007$).

Furthermore, to examine whether ASB16-AS1 was increased in CC cell lines, we determined its expression level in four CC cell lines: SiHa, HeLa, Ca-Ski and HT-3 cells via qRT-PCR. As shown in Fig. 1C, ASB16-

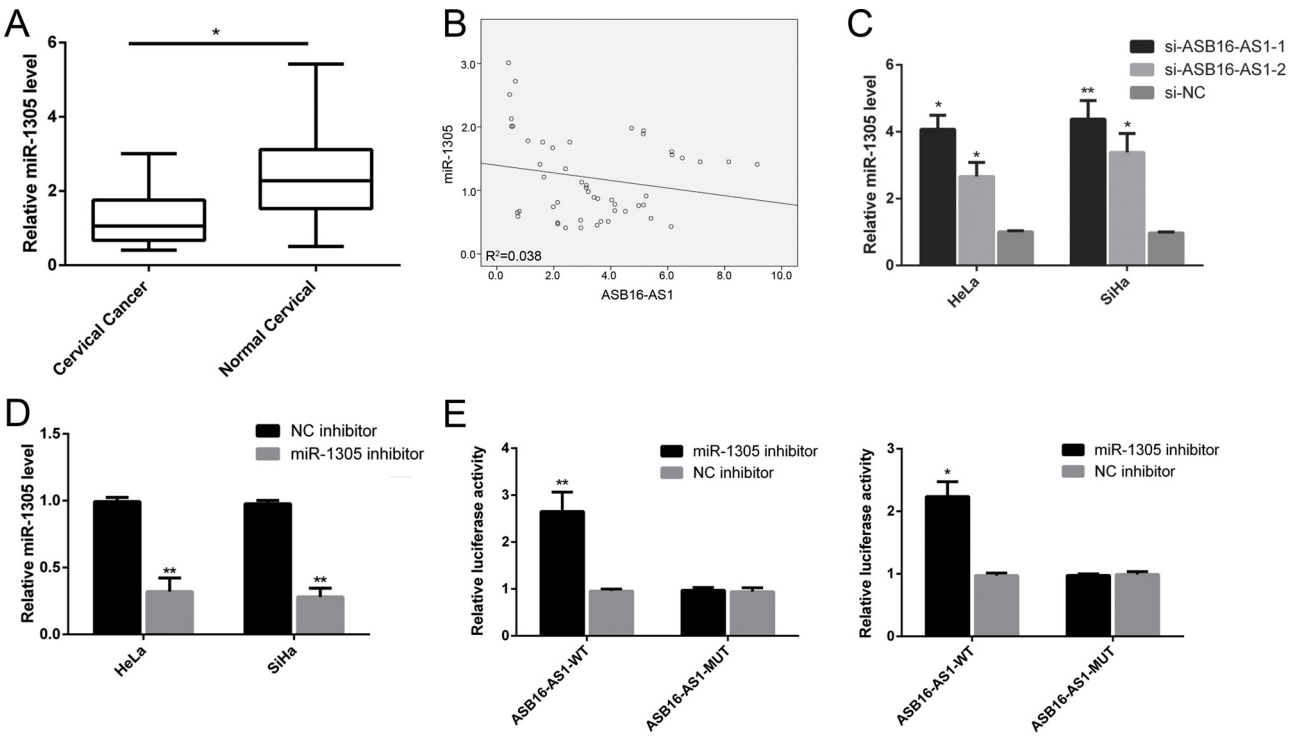

Fig. 3. ASB16-AS1 acts as a molecular sponge for miR-1305. **(a)** qRT-PCR was used to detect ASB16-AS1 expression in CC samples and normal samples(n = 60) **(b)** Association analysis of the relationship between ASB16-AS1 and miR-1305 expression levels in 60 paired CC tissues, data are subjected to Pearson correlation analysis(n = 60). **(c)** qRT-PCR was used to examine the expression of miR-1305 in HeLa and SiHa cells after transfection with si-ASB16-AS1-1 or si-ASB16-AS1-2 or si-NC. **(d)** qRT-PCR was used to examine the expression of miR-1305 in HeLa and SiHa cells after transfection with miR-1305 inhibitor or negative control. **(e)** The luciferase reporter plasmid containing wild-type (WT) or mutant (Mut) ASB16-AS1 was cotransfected into HeLa and SiHa cells with miR-1305 inhibitor in parallel with an empty plasmid vector. Date are shown as mean ± SD from three independent experiments, by Student's test. *P < 0.05; **P < 0.01.

AS1 was significantly increased in all CC cell lines, compared to non-carcinoma cervical epithelial cells Ect1/E6E7($P < 0.05$; Fig. 1C). HeLa and SiHa cell lines were selected for subsequent experiments to further assess the role of ASB16-AS1 in CC cell growth and tumor progression.

3.2. ASB16-AS1 effects on cell proliferation, migration, invasion in CC

To confirm molecular mechanism of ASB16-AS1 in CC, we constructed siRNA for down-regulated the level of ASB16-AS1. The knockdown efficiency of ASB16-AS1 in CC cells transfected with si-ASB16-AS1-1 and si-ASB16-AS1-2 was confirmed by qRT-PCR. ($P < 0.05$; Fig. 1D).

CCK-8 assays showed that knockdown of ASB16-AS1 decreased the ability of proliferation in CC cells ($P < 0.05$; Fig. 2A). To further study the effect of ASB16-AS1 on CC cell growth, we performed colony formation assays. As shown on Fig. 2B, the cells transfected with si-ASB16-AS1-1 and si-ASB16-AS1-2 formed significantly less colonies than cells from si-NC group. To examine the influence of ASB16-AS1 on cell migration and invasion ability in CC, transwell assay was performed. Consistent with the above data, we found that inhibition of ASB16-AS1 down-regulated the migration and the invasion capacities of HeLa and SiHa cells ($P < 0.05$; Fig. 2C-D).

3.3. ASB16-AS1 acts as ceRNA for miR-1305 in CC

Using the bioinformatics tool database, miR-1305, miR-647 and miR-1273a were predicted to bind to ASB16-AS1. Moreover, we used qRT-PCR to detect the expressions of these three miRNAs in CC cell lines following transfection with si-ASB16-AS1-1 or si-ASB16-AS1-2, miR-1305 exhibited the greatest change (P < 0.05; Fig. S1A). So we selected miR-1305 for further analysis. The site in miR-1305 binding to lncRNA ASB16-AS1 was identified (Fig.S1B).

We measured the level of miR-1305 in 60 human cervical tissues samples. As shown in Fig. 3A, miR-1305 was obviously down-regulated in CC tissues, compared with histologically normal cervical tissues ($P < 0.05$). Moreover, qRT-PCR analysis indicated that the ASB16-AS1 level was negatively correlated with that of miR-1305 level in CC tissues ($r^2 = 0.338$; $P = 0.034$; Fig. 3B). Knockdown of ASB16-AS1 significantly increased the level of miR-1305 in both CC cell lines ($P < 0.05$; Fig. 3C). According to obtained results, si-ASB16-AS1-1 has stronger ability to facilitate miR-1305 expression than si-ASB16-AS1-2. Collectively, these data demonstrated that miR-1305 is a direct target of ASB16-AS1 in CC.

To determine the effect of miR-1305 on CC development and progression, HeLa and SiHa cells were transfected with miR-1305 inhibitor and NC inhibitor, and qRT-PCR was applied to verify knockdown efficiency (P < 0.05; Fig. 3D). Additionally, luciferase reporter assay confirmed that in both HeLa and SiHa cells transfected with miR-1305 inhibitor, luciferase activity of ASB16-AS1-WT was significantly higher than it was in those cells within the control group. No effect on the luciferase activity of ASB16-AS1-MUT was observed (P < 0.05; Fig. 3E).

Taken together, ASB16-AS1 played a role in CC as a ceRNA of miR-1305.

3.4. MiR-1305 inhibits cell proliferation, migration, and invasion in CC

To further investigate the function of miR-1305 in CC, HeLa and SiHa cells were transfected with miR-1305 inhibitor. The effect of miR-1305 down-regulation on CC cell proliferation was measured by CCK-8 assay. As shown in Fig. 4A, miR-1305 knockdown increased cell growth rate in both HeLa and SiHa cells($P < 0.05$). Colony formation assays indicated that the ability to form colonies in the miR-1305 treated cells was significantly increased ($P < 0.05$; Fig. 4B). Also, knockdown of

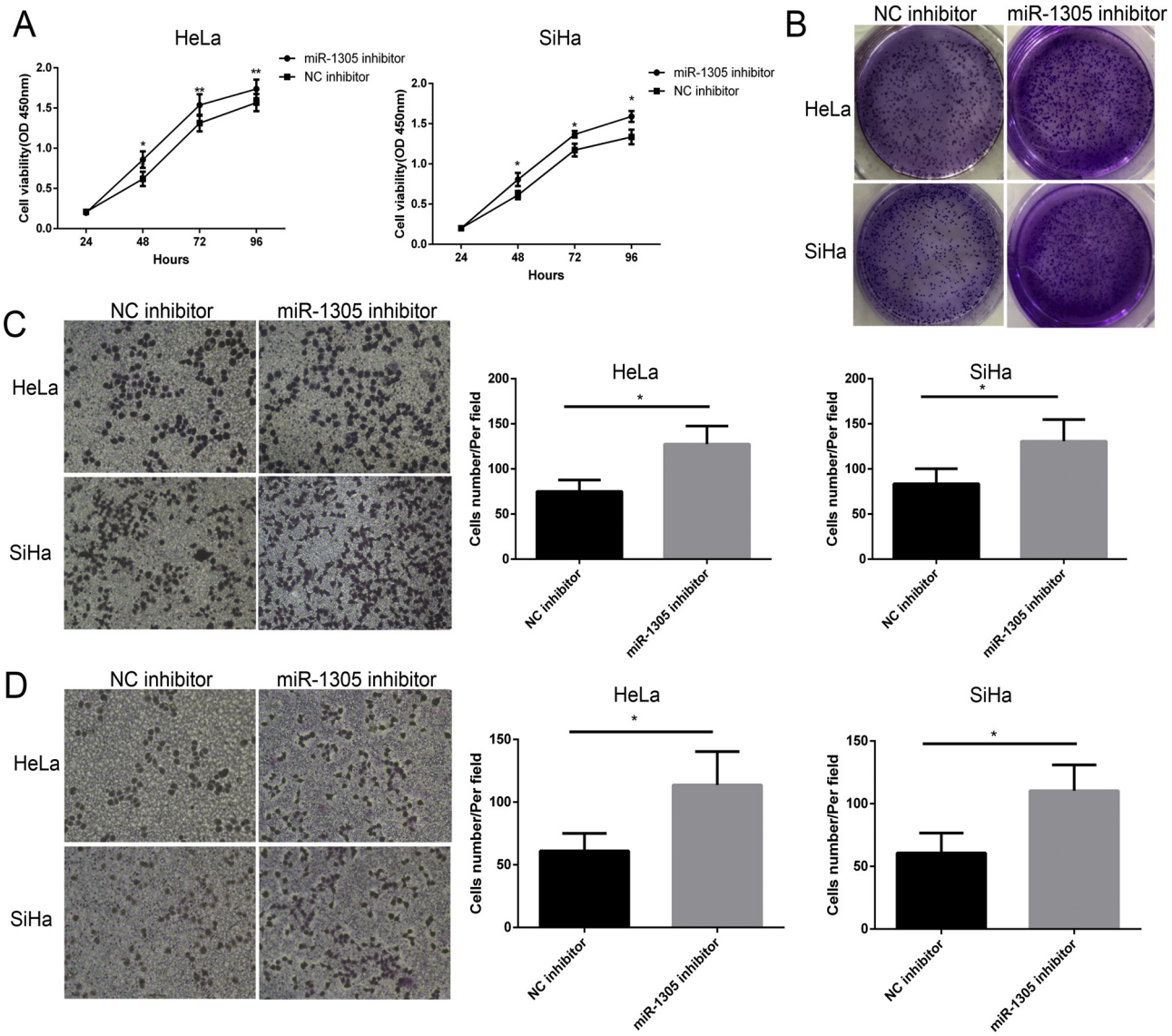

Fig. 4. miR-1305 inhibits cell proliferation, migration and invasion in CC cells. **(a)** CCK8 assays were used to determine the viability of miR-1305 inhibitor or NC-inhibtor transfected CC cells. **(b)** Colony formation assays were performed to determine the proliferation of miR-1305 inhibitor or NC-inhibtor transfected CC cells. The migration **(c)** and invasion **(d)** abilities of HeLa and SiHa cells were detected in transwell assays (without or with Matrigel) after transfection with miR-1305 inhibitor or NC-inhibtor. Date are shown as mean ± SD from three independent experiments, by Student's test. *P < 0.05;**P < 0.01.

miR-1305 increased the cell invasive and migratory capacity ($P < 0.05$; Fig. 4C, D).

Moreover, qRT-PCR was detected the expression of miR-505 in CC samples. As shown in Table2, the lower expression level of miR-505 was associated with advanced FIGO stage($P = 0.017$), larger tumor size ($P < 0.001$), positive lymph node metastasis($P = 0.004$), and poor histological grade($P = 0.032$) in CC patients.

3.5. ASB16-AS1 effects proliferation, migration, and invasion in CC cells by targeting miR-1305

To examine whether ASB16-AS1 exerts biological functions via miR-1305, we transfected HeLa and SiHa cells with si-ASB16-AS1-1 or miR-1305 inhibitor. CCK-8 assay and colony formation assay showed that cell proliferation and colony formation ability were markedly inhibited by silencing of ASB16-AS1 expression, whereas miR-1305 inhibition was found to partially antagonize this effect ($P < 0.05$; Fig. 5A-B). Furthermore, as shown in Fig. 5C-D, down- regulation of ASB16-AS1 could inhibit cell migration and invasion of CC cells, however, miR-1305 knockdown rescued the reduction of cell migration and invasion ($P < 0.05$). Taken together, these results indicated that ASB16-AS1 functioned as an oncogene by regulating miR-1305 in CC cells.

3.6. Wnt2 is a miR-1305 target gene and is indirectly regulated by ASB16-AS1

To investigate whether Wnt2 was a downstream target gene of miR-1305, western blot assay was performed. As demonstrated on Fig. 6A, knockdown of miR-1305 enhanced the expression levels of Wnt2 protein in both HeLa and SiHa cells($P < 0.05$). Luciferase reporter assay showed that the luciferase activity of the reporter containing Wnt2-WT was significantly increased in CC cells transfected with the miR-1305 inhibitor ($P < 0.05$; Fig. 6B), suggesting that Wnt2 was a miR-1305 target gene and indirectly regulated by ASB16-AS1. As lncRNA ASB16-AS1 could sponge miR-1305, we next determined whether ASB16-AS1 could regulate the expression of Wnt2 by binding to miR-1305. Western blot assays indicated that the expression of Wnt2 was decreased in si-ASB16-AS1-1 transfected CC cells, however, the inhibition of Wnt2

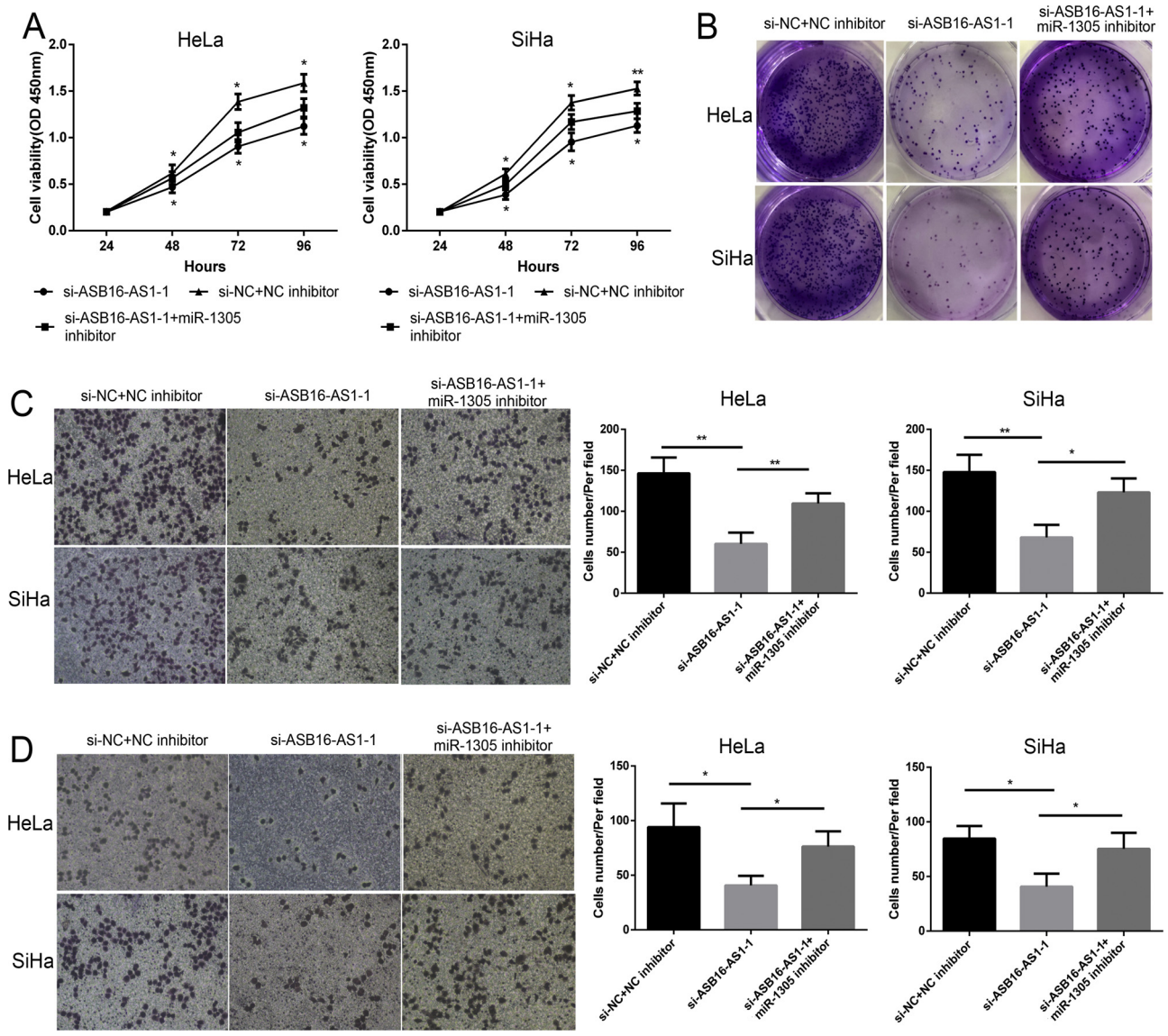

Fig. 5. ASB16-AS1 regulates cell proliferation, migration and invasion cells via miR-1305. **(a)** CCK8 assays were used to determine the viability of miR-1305 inhibitor or NC-inhibtor and si-ASB16-AS1-1 or si-NC co-transfected CC cells. **(b)** Colony formation assays were performed to determine the proliferation of miR-1305 inhibitor or NC-inhibtor and si-ASB16-AS1-1 or si-NC co-transfected CC cells. The cell migration **(c)** and invasion **(d)** abilities of HeLa and SiHa cells were detected in transwell assays (without or with Matrigel) after co-transfection with miR-1305 inhibitor or NC-inhibtor and si-ASB16-AS1-1 or si-NC. Date are shown as mean ± SD from three independent experiments, by Student's test. *P < 0.05;**P < 0.01.

protein levels induced by si-ASB16-AS1-1 was effectively reversed by miR-1305 inhibitor ($P < 0.05$; Fig. 6C)

3.7. ASB16-AS1 activates Wnt/β-catenin signaling pathway by targeting miR-1305 in CC cells

Wnt2 is considered as a regulator in Wnt/β-catenin signaling, so ASB16-AS1 may function as an oncogene by regulating Wnt/β-catenin signaling. To determine this hypothesis, so we measured the activity of Wnt signaling pathway. The results showed that the protein levels of Wnt/β-catenin signaling related proteins (nuclear β-catenin, c-Myc and p21) were decreased by ablation of ASB16-AS1, whereas the effect of ASB16-AS1 inhibition in HeLa and SiHa cells was significantly attenuated in the presence of miR-1305 inhibitor ($P < 0.05$; Fig. 7A, B). Collectively, our data reveal ASB16-AS1/miR-1305 could mediate the activity of Wnt/β-catenin signaling pathway in CC cells.

To further study the molecular mechanisms involved in the ASB16-AS1/miR-1305 regulated Wnt/β-catenin pathway, HeLa and SiHa cells were transfected with si-ASB16-AS1-1, treatment with or without CHIR-99,021 a kind of Wnt/β-catenin signaling pathway activator, then cell proliferation, colony formation, migration and invasion were determined by CCK8, colony formation, and transwell assays, respectively. As shown in Fig. 7C-D, knockdown of ASB16-AS1 weakened the cell proliferation rate, however, CHIR-99,021 was found to partially reverse this effect$P < 0.05$). Moreover, treatment by CHIR-99021 partially rescued the effect of ASB16-AS1 on migration and invasion, indicating that Wnt/β-catenin signaling pathway could be a downstream mechanism of ASB16-AS1 in CC ($P < 0.05$; Fig. 7E-F).

3.8. ASB16-AS1 knockdown effects cervical cancer tumorigenicity and metastasis in vivo

To determine the effect of ASB16-AS1 on CC tumorigenesis in vivo, lenti-si-NC or lenti-si-ASB16-AS1 transfected Hela stably were subcutaneously injected into nude mice. We found that the tumor volume and weight were significantly decreased in the lenti-si-ASB16-AS1

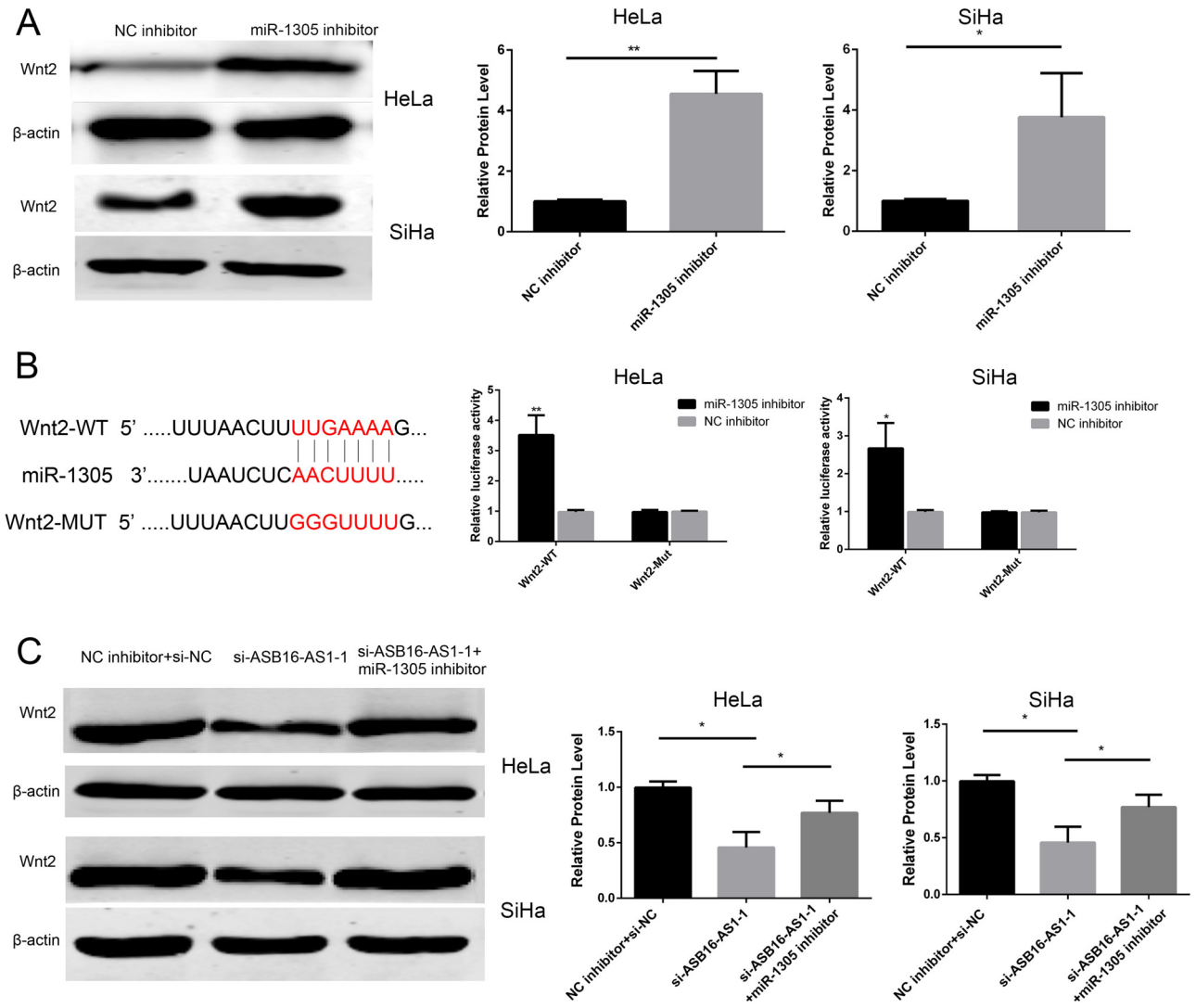

Fig. 6. Wnt2 is a target of miR-1305 and is suppressed by ASB16-AS1 deletion. **(a)** Protein level of Wnt2 in HeLa and SiHa cells transfected with miR-1305 inhibitor or NC-inhibitor. **(b)** Schematic view of miR-1305 targeting site in the WT and Mut 3′UTR of Wnt2.(left) Luciferase activity assay in HeLa and SiHa cells transfected with luciferase report plasmids containing Wnt2 3′UTR (WT or Mut), and miR-1305 inhibitor or NC-inhibitor. (right). (c) Wnt2 protein level in HeLa and SiHa cells cells following knockdown of ASB16-AS1 and/or inhibition of miR-1305. Date are shown as mean ± SD from three independent experiments, by Student's test. *P < 0.05;**P < 0.01.

group, compared with the lenti-si-NC group (Fig. 8 A, B). Results revealed that silencing of ASB16-AS1 suppressed tumor growth in vivo, implying ASB16-AS1 overexpression promotes the progression of CC.

We also measured the expression of ASB16-AS1 and miR-1305 by qRT-PCR analysis, the results showed that there was significantly downregulation the expression of ASB16-AS1 while upregulation the expression of miR-1305 in the lenti-si-ASB16-AS1 group compared to the lenti-si-NC group ($P < 0.01$; Fig. 8D-E). Furthermore, by performing western blot assays of the dissected tissues, ASB16-AS1 knockdown decreased the levels of Wnt2 *in vivo* ($P < 0.01$; Fig. 8F). Taken together, the aforementioned data confirms that he oncogenic activity of ASB16-AS1 in CC *in vivo*.

4. Discussion

Recently, the critical role of lncRNA in cancer progression and cancer metastasis has attracted many attentions. However, the molecular mechanisms for lncRNA functions in these pathological processes have not been explained clearly [14,15]. LncRNAs are recognized biomarkers of diagnosis, predictors of prognosis, and targets of treatment for CC [16,17]. In our study, we showed that the expression of ASB16-AS1 was increased in CC tissues and CC cell lines. Moreover, the higher level of ASB16-AS1 expression was associated with larger tumor size, positive lymph node metastasis, and poor histological grade in CC patients. These finding suggest that ASB16-AS1 functions as an oncogene and should be considered a novel therapeutic target in CC.

LncRNA ASB16-AS1 could promote cell proliferation, migration, and invasion in glioma [18]. However, the function of ASB16-AS is still largely unknown in CC. Our present findings first indicating knockdown of ASB16-AS1 could inhibit CC cell proliferation, migration, and invasion both *in vitro* and *in vivo*. Accumulating evidences are implied that lncRNAs are known to regulate gene expression through a series kind of methods. Among these mechanisms, lncRNAs mainly act as miRNA sponges by binding and triggering off their binding sites on protein-coding messengers to regulate downstream target genes. For example, lncRNA-AGAP2-AS1 was reported as an oncogene by absorbing miR-16 in HCC hepatocellular carcinoma (HCC) [19]. LncRNA CRNDE functioned as a sponge of miR-338 to promote cancer progression in non-small cell lung cancer (NSCLC) [20]. Combination with bioinformatics analyses and luciferase reporter assays, our results showed miR-1305 was binding to ABS16-AS1directly in CC cells [21]. Our data suggested miR-1305 exerted its suppressive effects on proliferation, migration,

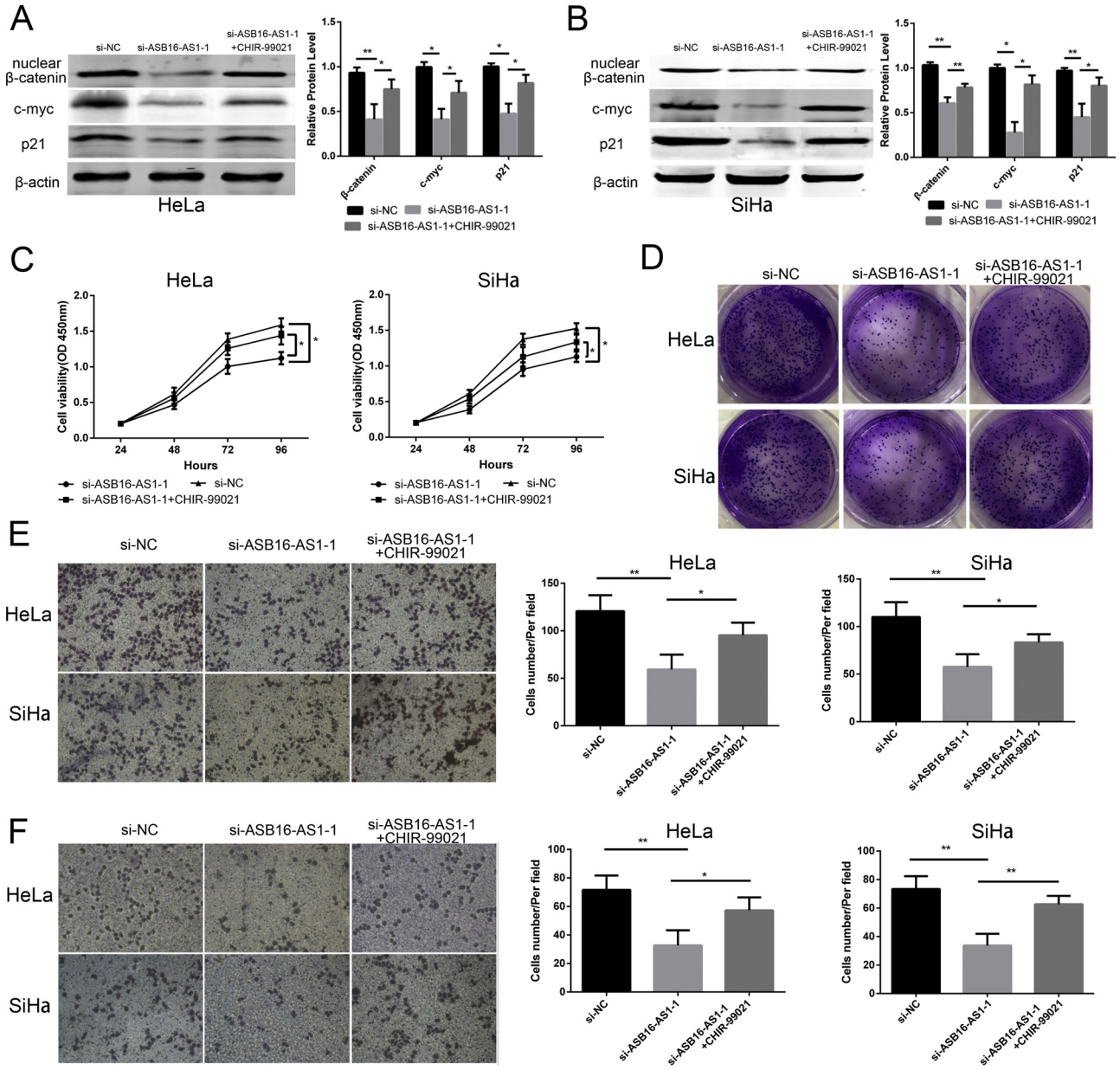

Fig. 7. ASB16-AS1 regulates the activity of Wnt/β-catenin via miR-1305 in CC cells. **(a)** Levels of Wnt/β-catenin marker proteins in both HeLa and SiHa cells after transfection with si-ASB16-AS1-1, treatment with or without CHIR-99,021. **(b)** CCK-8 assays were used to detect the viability of CC cells transfected with si-ASB16-AS1-1 or si-NC, in the presence or absence of CHIR-99,021. **(c)** Colony formation assays were performed to determine the proliferation of CC cells transfected with si-ASB16-AS1-1 or si-NC, in the presence or absence of CHIR- 99,021. The cell migration **(e)** and invasion **(f)** abilities of HeLa and SiHa cells were detected in transwell assays (without or with Matrigel) after transfection with si-ASB16-AS1-1 or si-NC, in the presence or absence of CHIR- 99021. Date are shown as mean ± SD from three independent experiments, by Student's test. *P < 0.05;**P < 0.01.

and invasion in CC cells. In addition, through the rescued experiment, depletion of miR-1305 reversed ASB16-AS1 affected cell growth and metastasis in CC, which suggested that miR-1305 antagonized the function of ASB16-AS1.

In general, lncRNAs function by acting as ceRNA depend upon modulation of miRNA targets; hence, miRNA target play is an important role in lncRNA-miRNA-mRNA network. Mature miRNAs have been confirmed to bind to the 3′UTR of target mRNAs, leading to suppression translation or induction of degradation of target mRNAs [22]. So far, a growing number of experiments have revealed that lncRNAs could modulate miRNAs target genes positively. For instance, Gao demonstrated that lncRNA MAGI1-IT1 could inhibit the expression of miR-200 in ovarian cancer [23]. We present evidence that miR-1305 was predicted to bind to Wnt2 by biological software analysis, moreover, luciferase reporter assay showed that the downregulation of miR-1305 increased the luciferase activity of Wnt2-WT 3′UTR. Taken together, all of our data showed ASB16-AS1 acted as a ceRNA to regulate the expression of the Wnt2, by sponging miR-1305 in CC.

Wnt2 is one of the widely studied members of the Wnt gene family, and plays tumorigenic roles in various types of cancers, including CC, HCC, ovarian cancer, et al [24–26]. Wnt2 binds to low-density lipoprotein receptor-related protein 5/6 co-receptors, then promotes the process of β-catenin translocated into the nucleus, results in activation Wnt/β-catenin signaling pathway [27]. Wnt/β-catenin signaling pathway in regulating tumorigenesis and metastasis is well studied [28]. Therefore, we speculated ASB16-AS1 could mediate the activity of Wnt/β-catenin signaling via Wnt2 in CC. Consistent with expected results, our study determined knockdown of ASB16-AS1 reduced the level

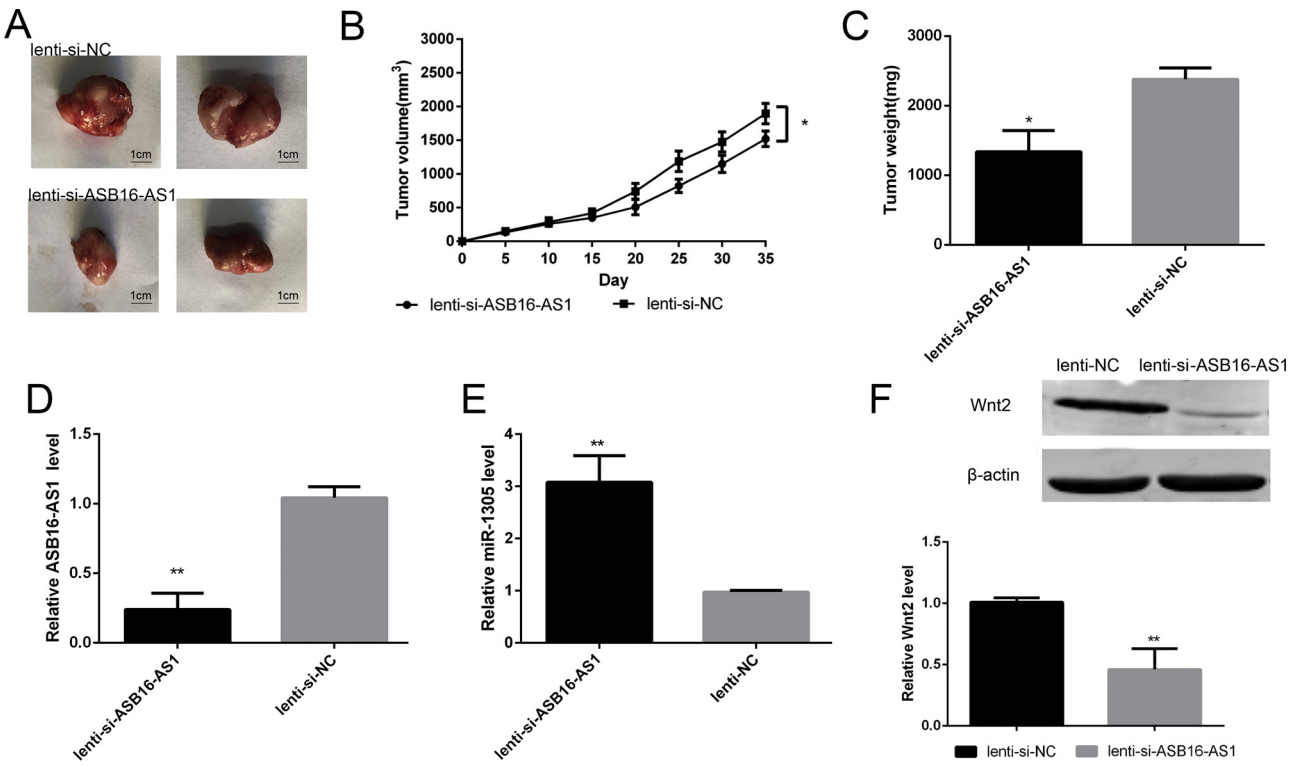

Fig. 8. ASB16-AS1 regulates tumor proliferation and progression in vivo. **(a)** The macroscopic xenograft tumors in nude mice from each group(n = 5). **(b)** Average tumor volumes were measured in xenograft mice every 5 days(n = 5). **(c)** The average xenograft tumor weight in nude mice from each group(n = 5). **(d)** Level of ASB16-AS1 was detected by qRT-PCR in tumor tissues from each group. **(e)** Level of miR-1305 was detected by qRT-PCR in tumor tissues from each group. **(f)** Wnt2 protein level were detected in tumor tissues in tumor tissues from each group by Western Blot. Date are shown as mean ± SD from three independent experiments, by Student's test. *P < 0.05;**P < 0.01.

of Wnt signaling pathway marker proteins. Moreover, treatment with CHIR-99,021, a kind of canonical Wnt signaling activator, abrogated the biological function of ASB16-AS1 in CC cells.

Our study has some limitations, the molecular mechanism of ASB16-AS1 in CC is still not full clearly. More cell lines or primary cell can be used to further verify whether ASB16-AS1 exerted similar function in cells with various kinds of molecular typing. Further studies should be conducted to unravel how ASB16-AS1 affects cell apoptosis, cell cycle, and the process of EMT.

5. Conclusions

In conclusion, our data showed that ASB16-AS1 is an oncogenic lncRNA in CC, and facilitates CC cell proliferation, colony formation, migration, and invasion by sponging miR-1305 both *in vitro* and *in vivo*. Wnt2 is a target gene of miR-1305 and mediates its biological effects through Wnt/β-catenin signal pathway. This study will improve understanding of mechanism involved in cancer progression and provide a potential therapeutic target for the molecular treatment of CC.

6. Ethical approval and consent to participate

All experiments were approved by the Ethics Review Committee of the Second Affiliated Hospital of Harbin Medical University.

7. Consent for publication

All authors consented to the publication of this manuscript.

8. Availability of supporting data

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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Author contributions

Wenhua Tan and Rujin Zhuang designed the research. Wei Liu, Shujun Feng, and Zhaoyang Jia performed cellular experiments and animal experiments. Elena Kapora performed the bioinfomatics analysis. Xiaoxu Bai provided the samples and carried out pathological analysis. Wei Liu and Rujin Zhuang wrote the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.109965.

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