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LncRNA CBR3-AS1 predicts a poor prognosis and promotes cervical cancer progression through the miR-3163/LASP1 pathway

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LncRNA carbonyl reductase antisense RNA 1 (CBR3-AS1) is increased in cervical cancer and predicts poor prognosis. This study aims to investigate the underlying mechanism of lncRNA CBR3-AS1 in cervical cancer. LncRNA CBR3-AS1 and LASP1 expressions were significantly elevated in cervical cancer tissue and cells, whereas miR-3163 expression was significantly decreased in cervical cancer tissue and cells. High lncRNA CBR3-AS1 expression and LASP1 expression showed a lower overall survival rate, whereas high miR-3163 expression showed a higher overall survival rate. Correlation between clinicopathological parameters of cervical cancer patients and lncRNA CBR3-AS1, miR-3163, LASP1 expressions indicated that the expressions of lncRNA CBR3-AS1, miR-3163, and LASP1 were closely related with distant metastasis and lymphatic metastasis of cervical cancer. LncRNA CBR3-AS1 knockdown suppressed cervical cancer cell viability and inhibited cancer stem cell-like properties. Besides, we identified that lncRNA CBR3-AS1 interacted with miR-3163, and miR-3163 targeted to LASP1. Moreover, the correlation between lncRNA CBR3-AS1 and miR-3163, as well as the correlation between miR-3163 and LASP1 was confirmed. Finally, lncRNA CBR3-AS1 knockdown inhibited tumor growth and suppressed cancer stem cell-like properties of cervical cancer in vivo. Taken together, high expression of lncRNA CBR3-AS1 predicts poor prognosis in cervical cancer, and the lncRNA CBR3-AS1/miR-3163/LASP1 pathway plays a vital function in the modulation of cervical cancer cell proliferation and cancer stem cell-like properties.

Key words: lncRNA CBR3-AS1, miR-3163, LASP1, proliferation, cancer stem cell-like properties, poor prognosis

Cervical cancer is the third malignant cancer in the female reproductive system which develops due to the persistent infection with high-risk human papillomavirus (HPV) [1]. According to the latest data from Cancer Statistics in 2020, the estimated number of new cases can reach 13,800 and the estimated deaths can reach 4,290 in the United States of America (USA) [2]. Although some treatments have been used to treat cervical cancer, including chemotherapy, radiation therapy, and surgery, the 5-year survival rate is not high and the prognosis is poor because of lymph node metastasis and distant metastasis [3, 4]. Cancer stem cells have stem cell-like properties characterized by the expression of stemness factors (NANOG, OCT4, and SOX2) which play a critical role in cervical cancer growth and metastasis [5, 6]. Therefore, there is an urgent need to focus on molecular targets of cervical cancer to control cervical cancer metastasis.

Mounting long non-coding RNAs (lncRNAs), such as lncRNA STXBP5-AS1, LINC00675, and PTENP1, facilitate or repress the proliferation, invasion, cancer stem cell-like properties, metastasis of cervical cancer [7–9]. LncRNA carbonyl reductase antisense RNA 1 (CBR3-AS1) is a widely expressed lncRNA in various cancers, such as breast cancer, osteosarcoma, and non-small cell lung cancer, which can promote tumorigenesis, proliferation, migration, and tumor growth of cancers [10–12]. However, the function of lncRNA CBR3-AS1 in regulating tumor growth and cancer stem cell-like properties of cervical cancer is not clear.

MicroRNA-3163 (miR-3163) is a miRNA that is related to the migration, proliferation, and tumor growth of cancer cells, such as cervical cancer, colorectal cancer, and non-small cell lung cancer [13–15]. Researchers have found that miR-3163 is downregulated in hepatocellular carcinoma and colorectal cancer, and the dysregulation of miR-3163 participated in the evolvement of hepatocellular carcinoma and colorectal cancer [15, 16]. Importantly, Yang et al. have discovered miR-3163 is decreased in cervical cancer cells, and dysregulation of miR-3163 can affect cervical cancer progression [13]. Although miR-3163 can inhibit or enhance the drug

resistance of retinoblastoma cancer stem cells or hepatocellular carcinoma cells [17, 18], the effect of miR-3163 in the regulation of cancer stem cell-like properties of cervical cancer is still unknown.

LIM And SH3 Protein 1 (LASP1) is a member of the nebulin family of actin-binding proteins which acts as a regulator of the growth and metastasis of cancers, such as head and neck squamous cell carcinoma, lung cancer, and breast cancer [19–21]. LASP1 can promote oncogenic processes and regulate lncRNAs and miRNAs-mediated glioma progression [22]. In addition, LASP1 plays critical functions in promoting the growth and migration of head and neck squamous cell carcinoma [21]. Importantly, LASP1 knockout decreased the expressions of Nanog, OCT4, and SOX2 in colorectal cancer cells, indicating LASP1 might be related to cancer stem cell-like properties [20].

In this study, we identified the function of lncRNA CBR3-AS1/miR-3163/LASP1 pathway in the modulation of cervical cancer cell proliferation and cancer stem cell-like properties. Besides, high lncRNA CBR3-AS1 expression showed a lower overall survival rate, and the expressions of lncRNA CBR3-AS1, miR-3163, and LASP1 were closely related to distant metastasis and lymphatic metastasis of cervical cancer, which may supply the theoretic basis for repressing cervical cancer.

Table 1. Correlation between CBR3-AS1 expression and clinicopathological parameters of CC patients.

Characteristics	Number	CBR3-AS1 expression		p-value
		High 28	Low 28	
Age (years)				0.778
<40	25	12	13	
≥40	31	16	15	
FIGO stage				0.016*
I–II	27	9	18	
III–IV	29	19	10	
Tumor size (cm)				0.422
≤4	29	13	16	
>4	27	15	12	
Distant metastasis				0.007*
Yes	30	20	10	
No	26	8	18	
Lymphatic metastasis				0.003*
Yes	31	21	10	
No	25	7	18	

Table 2. Correlation between miR-3163 expression and clinicopathological parameters of CC patients.

Characteristics	Number	miR-3163 expression		p-value
		High 28	Low 28	
Age (years)				0.420
<40	25	14	11	
≥40	31	14	17	
FIGO stage				0.001*
I–II	27	20	7	
III–IV	29	8	21	
Tumor size (cm)				0.789
≤4	29	15	14	
>4	27	13	14	
Distant metastasis				0.007*
Yes	30	10	20	
No	26	18	8	
Lymphatic metastasis				0.001*
Yes	31	21	9	
No	25	7	19	

Table 3. Correlation between LASP1 expression and clinicopathological parameters of CC patients.

Characteristics	Number	LASP1 expression		p-value
		High 28	Low 28	
Age (years)				0.179
<40	25	10	15	
≥40	31	18	13	
FIGO stage				0.181
I–II	27	11	16	
III–IV	29	17	12	
Tumor size (cm)				0.422
≤4	29	12	15	
>4	27	16	13	
Distant metastasis				0.032*
Yes	30	19	11	
No	26	9	17	
Lymphatic metastasis				0.016*
Yes	31	20	11	
No	25	8	17	

Patients and methods

Sample collection. Cervical cancer samples and adjacent normal tissues (56 pairs) were collected from cervical cancer patients when they underwent surgery for cervical cancer at The Affiliated Zhangjiagang Hospital of Soochow University and were confirmed by two histopathologists. Cervical cancer patients were divided into low- and high-lncRNA CBR3-AS1, miR-3163, and LASP1 groups, then Kaplan-Meier (K-M) survival curves were plotted using the survival data of cervical cancer patients. In Tables 1, 2, and 3, the correlation between clinicopathological parameters (age, FIGO stage, tumor size, distant metastasis, and lymphatic metastasis) of cervical cancer patients and lncRNA CBR3-AS1, miR-3163, LASP1 expressions were summarized. None of the patients had any therapy before the surgery. Written informed consent was obtained from all cervical cancer patients. This study was approved by the Ethics Committee of The Affiliated Zhangjiagang Hospital of Soochow University.

Cell lines and transfection. Human endocervical epithelial cell line End1/E6E7 cells (Huatuo Biotechnology Co., Ltd.), cervical cancer cell lines C-33A (Huatuo Biotechnology Co., Ltd.), SiHa (Shanghai Cell Bank of Chinese Academy of Sciences), CaSki (EK-Bioscience) and HeLa (Shanghai Cell Bank of Chinese Academy of Sciences) were kept in our laboratory. Cells were cultured in Minimum Essential Medium (MEM; Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, CA, USA), 100 μg/ml streptomycin, and 100 U/ml penicillin (Sigma-Aldrich, MO, USA) in a 5% CO_2 incubator at 37 °C.

si-CBR3-AS1#1 (small interfering RNA targeting CBR3-AS1; sequences, 5'-GTCTCCTGAGCTCAGGAAA-3'), si-CBR3-AS1#2 (sequences, 5'-GTCTGGCTAGAGGTTTACC-3'), si-CBR3-AS1#3 (sequences, 5'-AGCTCAAATTTTTTATATATTTC-3'), and siRNA negative control (si-NC), miR-3163 overexpressing vector (miR-3163) and negative control mimic (miR-NC), miR-3163 inhibitor and negative control inhibitor (NC inhibitor) were synthesized by GENECHEM (Shanghai, China). cDNA encoding LASP1 was inserted into the pcDNA plasmid (Invitrogen, CA, USA), and pcDNA (empty) negative control was used. Lipofectamine reagent (Invitrogen, CA, USA) was used for transfection.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The viability of SiHa and Hela cells was measured by MTT assay. SiHa and Hela cells with different transfection were seeded in 96-well plates at a concentration of 2×10^3/well. SiHa and Hela cells were cultured with MTT solution (10 μl; Beyotime Biotechnology, Nantong, China) for 4 h at room temperature. Formazan solution (100 μl) was added to cells and cultured at 37 °C for 2 h. The optical density (OD) value at 450 nm wavelength was determined by a microplate reader (Bio-Rad, CA, USA).

Colony formation assay. SiHa and Hela cells with different transfection were seeded into 6-well plates (400 cells/well). After two weeks, cells were fixed with paraformaldehyde (4%; Sigma-Aldrich, MO, USA) for 20 min, and then stained with crystal violet (0.1%; Sigma-Aldrich, MO, USA) for 30 min. Finally, images of visible colonies were photographed, and the colony areas were counted by ImageJ software.

Flow cytometric analysis for the detection of aldehyde dehydrogenase (ALDH) activity. SiHa and Hela cells (2.5×10^5) with different transfection were stained using ALDEFLUOR kit (STEMCELL Technologies, NC, USA) for 30 min at 37 °C. Specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) treated cells were as negative control. The percentage of ALDH-positive cells was determined by a flow cytometer (BD, NJ, USA) with 488 nm blue laser and standard FITC 530/30 nm bandpass filter.

qRT-PCR. The total RNAs from cervical cancer tissues and cells were isolated by TRIzol reagent (Ambion). cDNA was synthesized by reverse transcription kit (PrimeScript IV 1st strand cDNA Synthesis Mix; Takara Bio, Dalian, China). The reactions of qRT-PCR were carried out using EXPRESS One-Step SYBR GreenER Kit (Invitrogen, CA, USA) in a CFX384 Touch Real-Time PCR System (Bio-rad, CA, USA). Relative gene expressions of CBR3-AS1, miR-3163, and LASP1 were normalized to GAPDH or U6 and measured by the comparative Ct method. The gene-specific primers sequences are used as the following: CBR3-AS1 F, 5'-CTGTCGCCCAGGCTGGAGTGC-3', R, 5'-GACGCCGTGGGTCCTTCTCATC-3'; miR-3163 F, 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGAAGGGTTG-3', R, 5'-ACACTCCAGCTGGGACAAAAAAAAAAGCCCA-3'; LASP1 F, 5'-CTGTCTCTGCCTTATAGCAACAC-3', R, 5'-CATCTCGAACCTGGCTGTTTG-3'; GAPDH F, 5'-AATCCCATCACCATCTTCCAG-3', R, 5'-GAGCCCCAGCCTTCTCCAT-3'; U6 F, 5'-CTCGCTTCGGCAGCACATATACT-3', R, 5'-CGCTTCACGAATTTGCGTGT-3'.

Western blotting. Proteins from cervical cancer tissues and cells were isolated using the RIPA kit (Sigma-Aldrich, MO, USA). An amount of 50 μg protein was separated on SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, MA, USA). Then, the membranes were incubated with anti-Ki-67 (1:1000; Abcam, Cambridge, UK; cat. number: ab231172), anti-PCNA (1:1500; Cell Signaling Technology, Boston, USA; cat. number: 2586), anti-OCT4 (1:1000; Cell Signaling Technology, Boston, USA; cat. number: 4286), anti-Nanog (1:1000; Cell Signaling Technology, Boston, USA; cat. number: 4893), anti-LASP1 (1:10000; Abcam, Cambridge, UK; cat. number: ab156872), anti-β-actin (1:2500; Cell Signaling Technology, Boston, USA; cat. number: 3700) for 12 hours at 4 °C, and then incubated with secondary antibody (1:3000; Cell Signaling Technology, Boston, USA; cat. number: 7076). The HRP-ECL method was applied, and the number of individual proteins was quantified by iBright Imaging System (ThermoFisher Scientific, CA, USA).

RNA pull-down assay. SiHa and HeLa cells were transfected with a biotin-labeled miR-3163 probe (Bio-miR-3163) or negative control probe (Bio-NC) [23]. SiHa and HeLa cells were collected and lysed using RIPA lysis and extraction buffer (ThermoFisher Scientific, CA, USA) after 48 h. Cell lysates were then incubated with Streptavidin magnetic beads (Pierce, CA, USA) for 12 h at 4 °C. After elution of Streptavidin magnetic beads, the bound RNAs were detected using qRT-PCR.

RNA immunoprecipitation (RIP) assay. SiHa and HeLa cells (2×10^7) were collected to perform RIP assay using an AGO2 antibody (Millipore, MA, USA). AGO2 antibody (5 μg; ThermoFisher Scientific, CA, USA; catalog number: MA5-23515) for each RIP was used in the RIP assay, and normal rabbit IgG was used as a negative control. Co-precipitated RNAs were isolated and detected by qRT-PCR.

Dual luciferase reporter gene assay. Dual-Luciferase Reporter Assay System (Promega, WI, USA) was used to measure the luciferase activity. The sequences of CBR3-AS1 or LASP1 3'-UTR were sub-cloned into pGL3 vectors. Then, SiHa and HeLa cells were cultured with wide type CBR3-AS1 vector (CBR3-AS1-WT) or mutant CBR3-AS1

vector (CBR3-AS1-MUT), wide type 3'-UTR of LASP1 vector (LASP1 3'UTR-WT) or mutant 3'-UTR of LASP1 vector (LASP1 3'UTR-MUT) using Lipofectamine 2000 reagent. Finally, relative luciferase activity was measured with Varioskan Lux Detection System (Thermo Scientific, CA, USA) (Supplementary data S1).

Xenograft mouse model. Female BALB/c nude mice (five-week-old) were obtained from Charles River (Beijing, China). HeLa cells (1×10^6/ml) suspended in PBS (0.1 ml HeLa cell suspension) were injected into the backs of nude mice subcutaneously. Mice were assigned into two groups, namely sh-NC and sh-CBR3-AS1 groups, with five mice in each group. We recorded tumor volumes every seven days and calculated by the formula: tumor volume [mm^3] = 0.5 × length × width^2). Thirty-five days later, all mice were sacrificed, and the tumors were excised, weighed, and used for further molecular analysis. This animal experiment was aproved by the Animal Care and Use Committee of The Affiliated Zhangjiagang Hospital of Soochow University.

Immunohistochemistry (IHC) analysis. Tumor tissue sections were de-paraffinized and rehydrated, added with 3% H_2O_2 for 30 min, and blocked with 1% FBS for 60 min, incubated with anti-Ki67 (1:200; Cell Signaling Technology, Boston, USA; cat. number: 12202), anti-PCNA (1:4000; Cell Signaling Technology, Boston, USA; cat. number: 13110), anti-OCT4 (1:100; Abcam, Cambridge, UK; cat. number: ab203859), anti-Nanog (1:500; Abcam, Cambridge, UK; cat. number: ab214549) at 4 °C for 12 h. Then, sections were incubated with secondary antibodies (1:1000; Abcam, Cambridge, UK; cat. number: ab6728) at 25 °C for 1 h. Then, tumor sections were stained with 3,3'-diaminobenzidine (DAB) staining solution and observed by a fluorescence microscope (Nikon, Tokyo, Japan).

Statistical analysis. All experiments were independently repeated three times. SPSS 17.0 software was applied to analyze all data, and the data were expressed as mean ± standard deviation (SD). Student's t-test or one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was employed to analyze the difference between different groups. Overall survival rates were estimated by K-M analysis, and log-rank test was performed for comparison. A p-value <0.05 was considered as the criterion of statistical significance.

Results

LncRNA CBR3-AS1 was elevated in cervical cancer tissue and cervical cancer cells and predicted a lower overall survival rate. To investigate whether there was a correlation between LncRNA CBR3-AS1 expression and the clinical prognosis of cervical cancer patients, 56 cervical cancer patients who underwent surgery were recruited. LncRNA CBR3-AS1 expression was significantly increased in cervical cancer tissue than in adjacent normal tissue (N=56; Figure 1A). Besides, the low lncRNA CBR3-AS1 expression group had a significantly higher overall survival rate than that of the high lncRNA CBR3-AS1 expression group (N=28; Figure 1B). The correlation between CBR3-AS1 expression and clinicopathological parameters of cervical cancer patients is shown in Table 1, indicating that CBR3-AS1 expression was closely related to FIGO stage, distant metastasis, and lymphatic metastasis of cervical cancer. Also, lncRNA CBR3-AS1 expression was significantly increased in cervical cancer cells (C-33A, SiHa, Caski, and HeLa) than End1/E6E7 cells (Figure 1C). These results indicated that high lncRNA CBR3-AS1 expression predicted a lower overall survival rate in cervical cancer.

LncRNA CBR3-AS1 knockdown reduced cervical cancer cell proliferation and inhibited cancer stem cell-like properties. To examine the function of lncRNA CBR3-AS1 in cervical cancer cells, CBR3-AS1 was inhibited in SiHa and HeLa cells. After the transfection of si-CBR3-AS1 (si-CBR3-AS1#1, si-CBR3-AS1#2, si-CBR3-AS1#3), lncRNA CBR3-AS1 expression was significantly decreased in SiHa and HeLa cells (Figure 2A). Also, lncRNA CBR3-AS1 expression was significantly decreased in End1/E6E7 cells (Supplementary Figure S1A). SiHa and HeLa cell viability was significantly reduced in the si-CBR3-AS1 group than in the si-NC

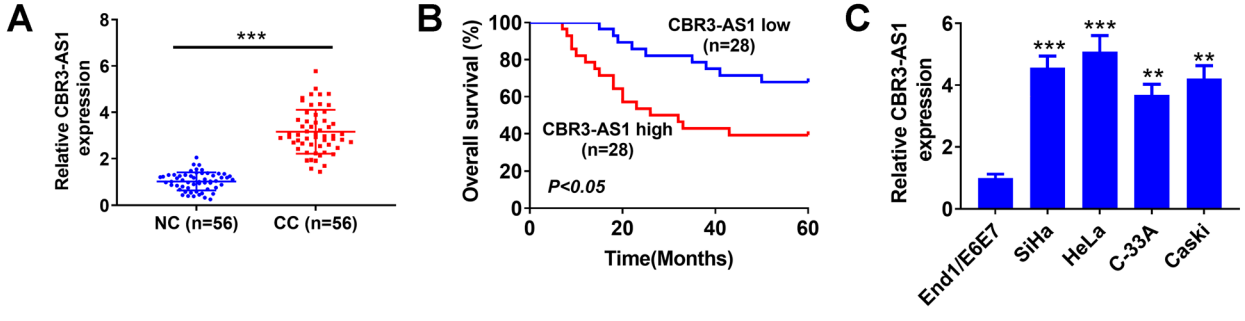

Figure 1. LncRNA CBR3-AS1 was elevated in cervical cancer tissue and cervical cancer cells. A) LncRNA CBR3-AS1 expression in cervical cancer tissue and adjacent normal tissue was measured using qRT-PCR. N=56. B) Overall survival rate of the low lncRNA CBR3-AS1 expression group and the high lncRNA CBR3-AS1 expression group (N=28). C) LncRNA CBR3-AS1 expression in cervical cancer cells (C-33A, SiHa, CaSki, and HeLa) and End1/E6E7 cells were detected using qRT-PCR. **p<0.01; ***p<0.001

groups (Figure 2B). The colony formation ability of SiHa and HeLa cells was significantly inhibited in si-CBR3-AS1 group than si-NC group (Figure 2C). However, End1/E6E7 cell viability and colony formation ability were not significantly changed in the si-CBR3-AS1 group than in the si-NC group (Supplementary Figures S1B, S1C). ALDEFUOR assay of SiHa and HeLa cells showed a significantly lower percentage of ALDH-positive cells in the si-CBR3-AS1 group than in the si-NC group (Figure 2D). Besides, the expressions of proliferation markers (Ki-67 and PCNA) and stem cell markers (OCT4 and Nanog) in SiHa and HeLa cells were significantly decreased in the si-CBR3-AS1 group than in the si-NC

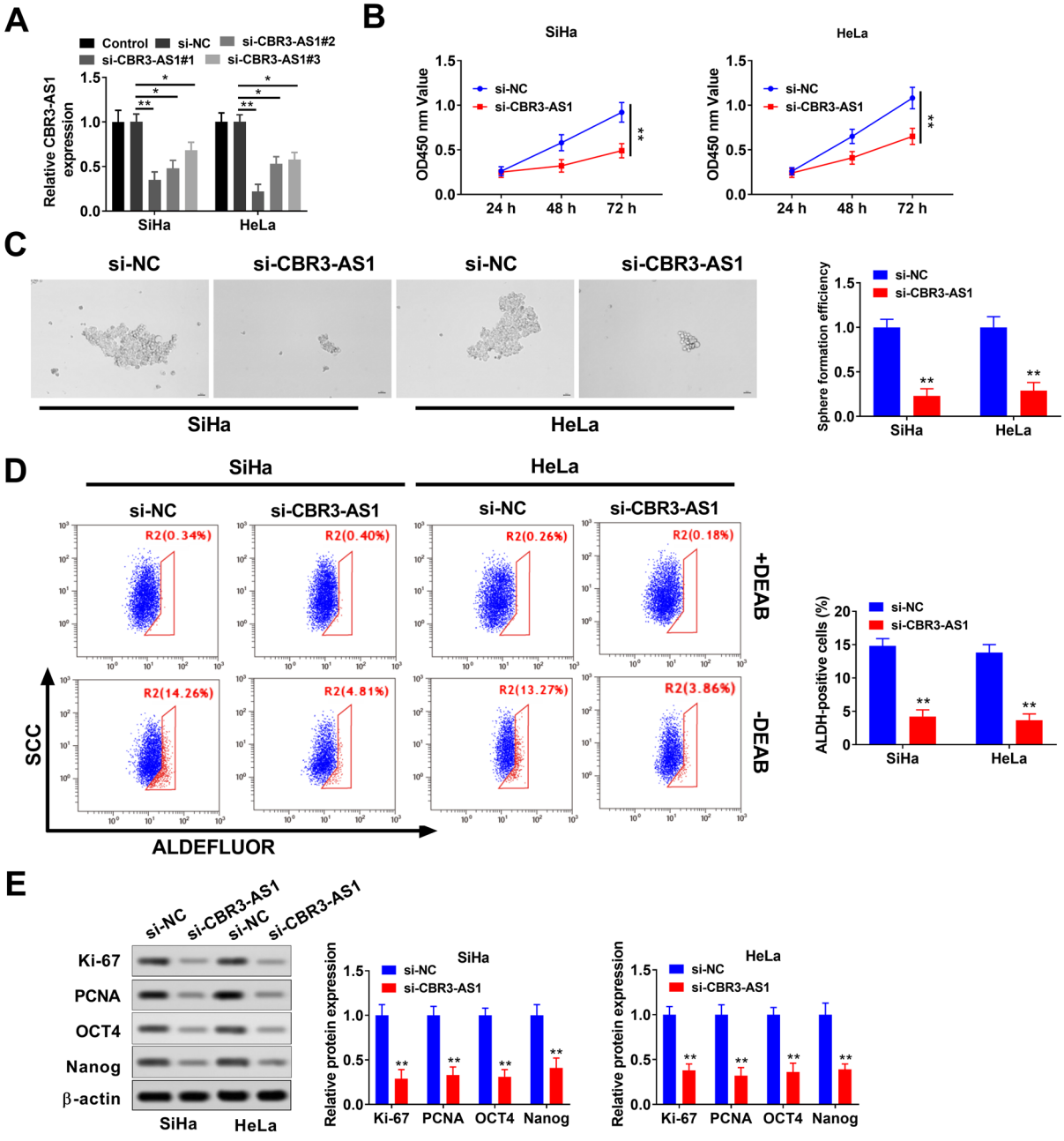

Figure 2. LncRNA CBR3-AS1 knockdown reduced cervical cancer cell proliferation and inhibited cancer stem cell-like properties. A) SiHa and HeLa cells were divided into control, si-NC, si-CBR3-AS1#1, si-CBR3-AS1#2, and si-CBR3-AS1#3 groups. LncRNA CBR3-AS1 expression was measured using qRT-PCR. B) SiHa and HeLa cells were divided into si-NC and si-CBR3-AS1 groups. Cell viability was measured using an MTT assay. C) Colony formation ability of SiHa and HeLa cells was measured using a colony formation assay. D) The percentage of ALDH-positive cells in the si-CBR3-AS1 group and the si-NC group was measured using the ALDEFUOR assay. E) Proliferation markers (Ki-67 and PCNA) and stem cell markers (OCT4 and Nanog) expressions in the si-CBR3-AS1 group and the si-NC group were measured using western blotting. *p<0.05; **p<0.01

groups (Figure 2E). These results indicated that lncRNA CBR3-AS1 facilitated cervical cancer cell proliferation and cancer stem cell-like properties.

CBR3-AS1 interacted with miR-3163, and high miR-3163 expression predicted a higher overall survival rate. To confirm whether CBR3-AS1 can interact with the target gene miR-3163, luciferase reporter gene, RNA pull-down, and RIP assays were conducted. The binding sites between CBR3-AS1 and miR-3163 were shown in Figure 3A. The luciferase activity of CBR3-AS1-WT was significantly inhibited by miR-3163, whereas the luciferase activity of CBR3-AS1-MUT was not changed by miR-3163 in SiHa and HeLa cells (Figure 3B). Compared with the bio-NC group, CBR3-AS1 can be enriched by bio-miR-3163 in SiHa and HeLa cells, and the biotinylated RNA pull-down also pulls down the AGO2 complex (Figure 3C). Compared with the anti-IgG group, CBR3-AS1 was enriched in the anti-AGO2 group in SiHa and HeLa cells, indicating CBR3-AS1 can bind with AGO2 (Figures 3D, 3E). After transfection of miR-3163 and followed by AGO2 pull down, the CBR3-AS1 enrichment in AGO2 was significantly regulated by miR-3163 (Figure 3F). In addition, miR-3163 expression was significantly decreased in cervical cancer tissue than in adjacent normal tissue (N=56; Figure 3G). The correlation between miR-3163 expres-

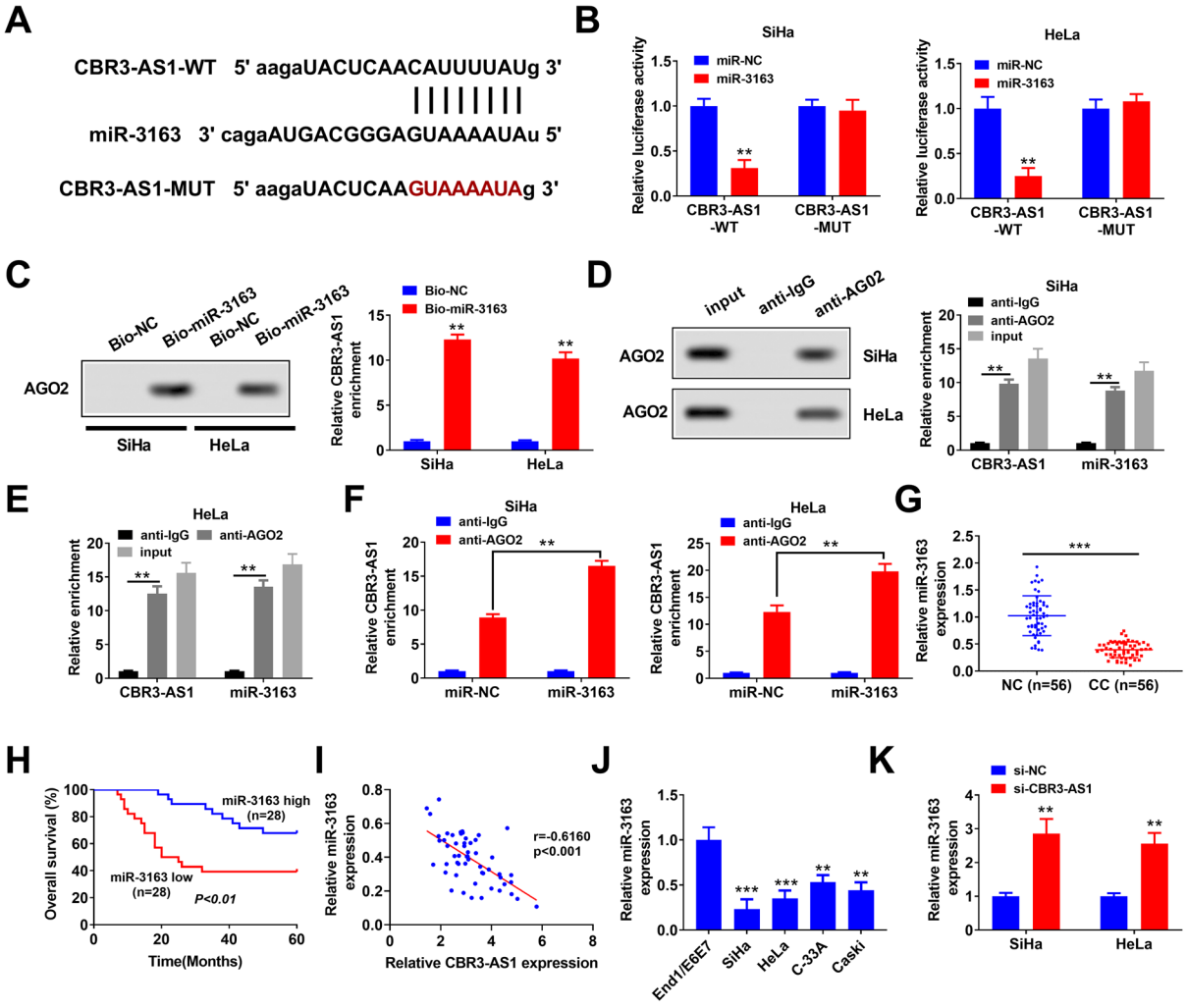

Figure 3. CBR3-AS1 interacted with miR-3163. A) The binding sites between CBR3-AS1 and miR-3163. B) CBR3-AS1 interacted with miR-3163 detected by dual-luciferase reporter gene assay. C) The enrichment of CBR3-AS1 by miR-3163 in SiHa and HeLa cells was detected using RNA pull-down assay. AGO2 complex was detected in the bio-NC and bio-miR-3163 groups by western blotting. D, E) Binding between CBR3-AS1 and AGO2 was determined by the RIP assay. AGO2 pull-down efficiency was validated by western blotting. F) After transfection of miR-3163 and followed by AGO2 pull down, the CBR3-AS1 enrichment in AGO2 was detected in the miR-NC and miR-3163 groups. G) miR-3163 expression in cervical cancer tissue and adjacent normal tissue was measured using qRT-PCR. N=56. H) Overall survival rate of the high miR-3163 expression group and low miR-3163 expression group. N=28. I) Correlation analysis of CBR3-AS1 and miR-3163. J) miR-3163 expression in cervical cancer cells (C-33A, SiHa, CaSki, and HeLa) and End1/E6E7 cells was detected by qRT-PCR. K) SiHa and HeLa cells were divided into si-NC and si-CBR3-AS1 groups. miR-3163 expression in the two groups was detected using qRT-PCR. **p<0.01; ***p<0.001

sion and clinicopathological parameters of cervical cancer patients was shown in Table 2, indicating that miR-3163 expression was closely related to FIGO stage, distant metastasis, and lymphatic metastasis of cervical cancer. The high miR-3163 expression group had a significantly higher overall survival rate than that of the low miR-3163 expression group (N=28; Figure 3H). Moreover, correlation analysis showed that there was a negative correlation between CBR3-AS1 and miR-3163 (Figure 3I). Also, miR-3163 expression was significantly decreased in cervical cancer cells (C-33A, SiHa, CaSki, and HeLa) than End1/E6E7 cells (Figure 3J). Further transfection experiments showed that si-CBR3-AS1 significantly upregulated miR-3163 expression (Figure 3K). These results indicated that CBR3-AS1 could interact with miR-3163, and low miR-3163 expression predicted a lower overall survival rate.

miR-3163 targeted to LASP1, and high LASP1 expression predicted lower overall survival rate. To further confirm whether miR-3163 can target to LASP1, LASP1 3'UTR-WT or LASP1 3'UTR-MUT was cloned into pGL3 vectors. Bioinformatics software predicted the binding sites between miR-3163 and LASP1 (Figure 4A). The luciferase activity of LASP1-3'UTR-WT was significantly decreased by miR-3163, whereas the luciferase activity of LASP1-3'UTR-MUT was not changed by miR-3163 (Figure 4B). Further transfection experiments showed that the miR-3163 overexpressing vector significantly upregulated miR-3163 expression, whereas the miR-3163 inhibitor significantly down-regulated miR-3163 expression in SiHa and HeLa cells (Figure 4C). miR-3163 overexpressing vector significantly decreased LASP1 expression, and miR-3163 inhibitor significantly upregulated LASP1 expression in SiHa and HeLa cells (Figure 4D). LASP1 expression was significantly elevated in cervical cancer tissue than in adjacent normal tissue (N=56; Figure 4E). The correlation between LASP1 expression and clinicopathological parameters of cervical cancer patients was shown in Table 3, indicating that LASP1 expression is closely related to distant metastasis and lymphatic metastasis of cervical cancer. The low LASP1 expression group had a significantly higher overall survival rate than that of the high LASP1 expression group (N=28; Figure 4F). Correlation analysis showed that there was a negative correlation between LASP1 and miR-3163 (Figure 4G), and there was a positive correlation between LASP1 and CBR3-AS1 (Figure 4H).

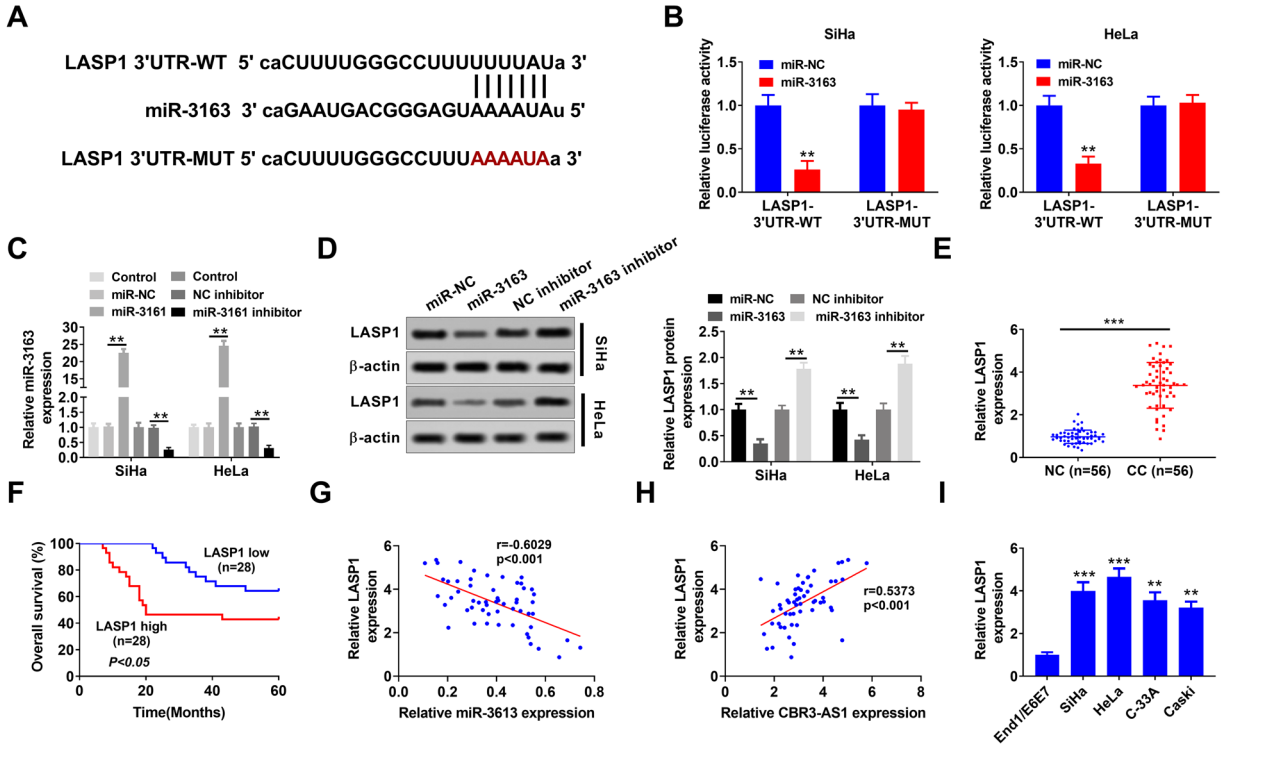

Figure 4. miR-3163 targeted to LASP1. A) The binding sites between miR-3163 and LASP1. B) miR-3163 targeted to LASP1 was determined using a dual-luciferase reporter gene assay. C) SiHa and HeLa cells were divided into control, miR-NC, miR-3163, control, NC inhibitor, and miR-3163 inhibitor groups. miR-3163 expression was measured using qRT-PCR. D) SiHa and HeLa cells were divided into the miR-NC, miR-3163, NC inhibitor, and miR-3163 inhibitor groups. LASP1 expression was measured using western blotting. E) The expression of LASP1 in cervical cancer tissue and adjacent normal tissue was measured using qRT-PCR. N=56. F) Overall survival rate of low LASP1 expression group and high LASP1 expression group. N=28. G) Correlation analysis of LASP1 and miR-3163. H) Correlation analysis of LASP1 and CBR3-AS1. I) LASP1 expression in cervical cancer cells (C-33A, SiHa, CaSki, and HeLa) and End1/E6E7 cells was detected using qRT-PCR. **p<0.01; ***p<0.001

Also, LASP1 expression was significantly increased in cervical cancer cells (C-33A, SiHa, CaSki, and HeLa) than End1/E6E7 cells (Figure 4I). These results indicated that miR-3163 could target to LASP1, and high LASP1 expression predicted a lower overall survival rate.

LncRNA CBR3-AS1 knockdown reduced cervical cancer cell proliferation and inhibited cancer stem cell-like properties through miR-3163. We further investigated whether the function of lncRNA CBR3-AS1 in cervical cancer cells was modulated by miR-3163 and LASP1.

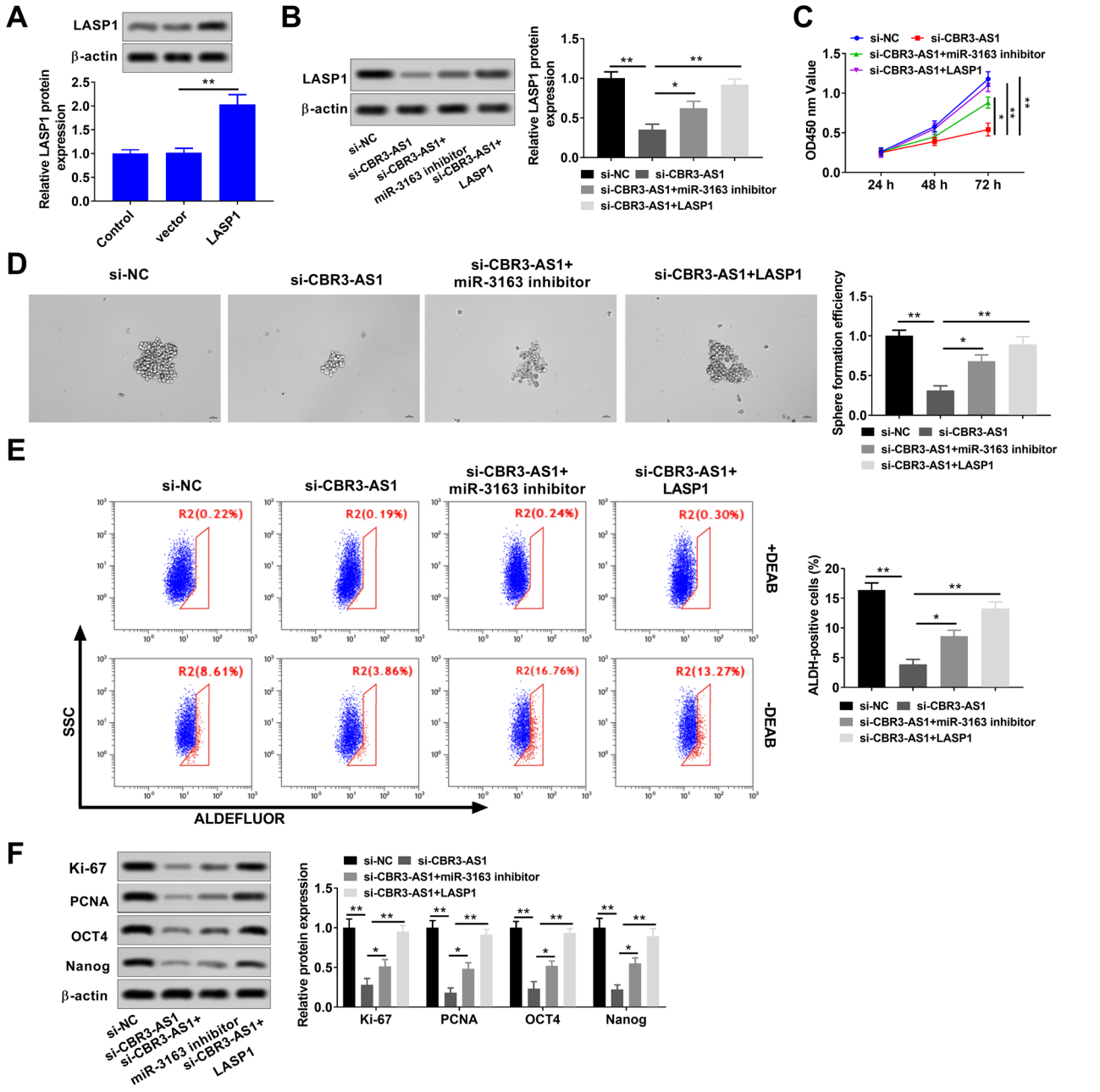

Figure 5. LncRNA CBR3-AS1 knockdown reduced cervical cancer cell proliferation and inhibited cancer stem cell-like properties through miR-3163. A) LASP1 protein expression in control, vector, and LASP1 overexpressing vector groups was measured by western blotting. B) There were four groups, namely si-NC, si-CBR3-AS1, si-CBR3-AS1+miR-3163 inhibitor, and si-CBR3-AS1+LASP1 groups. LASP1 protein expression in the four groups was detected by western blotting. C) Cell viability in the four groups was measured using an MTT assay. D) Colony formation ability in the four groups was measured using a colony formation assay. E) ALDH-positive cells (%) in the four groups were measured using ALDEFUOR assay. F) Proliferation markers (Ki-67 and PCNA) and stem cell markers (OCT4 and Nanog) expressions in the four groups were measured using Western blotting. *p<0.05; **p<0.01; ***p<0.001

Compared with the control and vector group, LASP1 expression was significantly increased in the LASP1 overexpressing vector group (Figure 5A). In addition, si-CBR3-AS1 significantly downregulated LASP1 expression, miR-3163 inhibitor abolished the suppressive effect of si-CBR3-AS1 on LASP1 expression, and LASP1 overexpressing vector also abolished the suppressive effect of si-CBR3-AS1 on LASP1 expression (Figure 5B). si-CBR3-AS1 significantly reduced cell viability, miR-3163 inhibitor abolished the suppressive effect of si-CBR3-AS1 on cell viability, and LASP1 overexpressing vector also abolished the suppressive effect of si-CBR3-AS1 on cell viability (Figure 5C). si-CBR3-AS1 significantly repressed cell proliferation, miR-3163 inhibitor abolished the suppressive effect of si-CBR3-AS1 on cell proliferation, and LASP1 overexpressing vector also abolished the suppressive effect of si-CBR3-AS1 on cell proliferation (Figure 5D). si-CBR3-AS1 decreased the percentage of ALDH-positive cells, miR-3163 inhibitor abolished the suppressive effect of si-CBR3-AS1 on cancer stem cell-like properties, and LASP1 overexpressing vector also abolished the suppressive effect of si-CBR3-AS1 on cancer stem cell-like properties (Figure 5E). si-CBR3-AS1 down-regulated proliferation markers (Ki-67 and PCNA) and stem cell markers (OCT4 and Nanog) expressions, miR-3163 inhibitor abolished the suppressive effect of si-CBR3-AS1 on the expressions of these proteins, and LASP1 overexpressing vector also abolished the suppressive effect of si-CBR3-AS1 on the expressions of these proteins (Figure 5F). These results indicated that lncRNA CBR3-AS1 facilitated cervical cancer cell proliferation and cancer stem cell-like properties via miR-3163.

LncRNA CBR3-AS1 knockdown repressed tumor growth and suppressed cancer stem cell-like properties of cervical cancer *in vivo*. To test the role of lncRNA CBR3-AS1 *in vivo*, HeLa cells were subcutaneously injected into the backs of nude mice. Tumor volume and weight were significantly suppressed in the sh-CBR3-AS1 group than in the sh-NC group (Figures 6A–6C). CBR3-AS1 and LASP1 RNA expressions were significantly decreased in the sh-CBR3-AS1

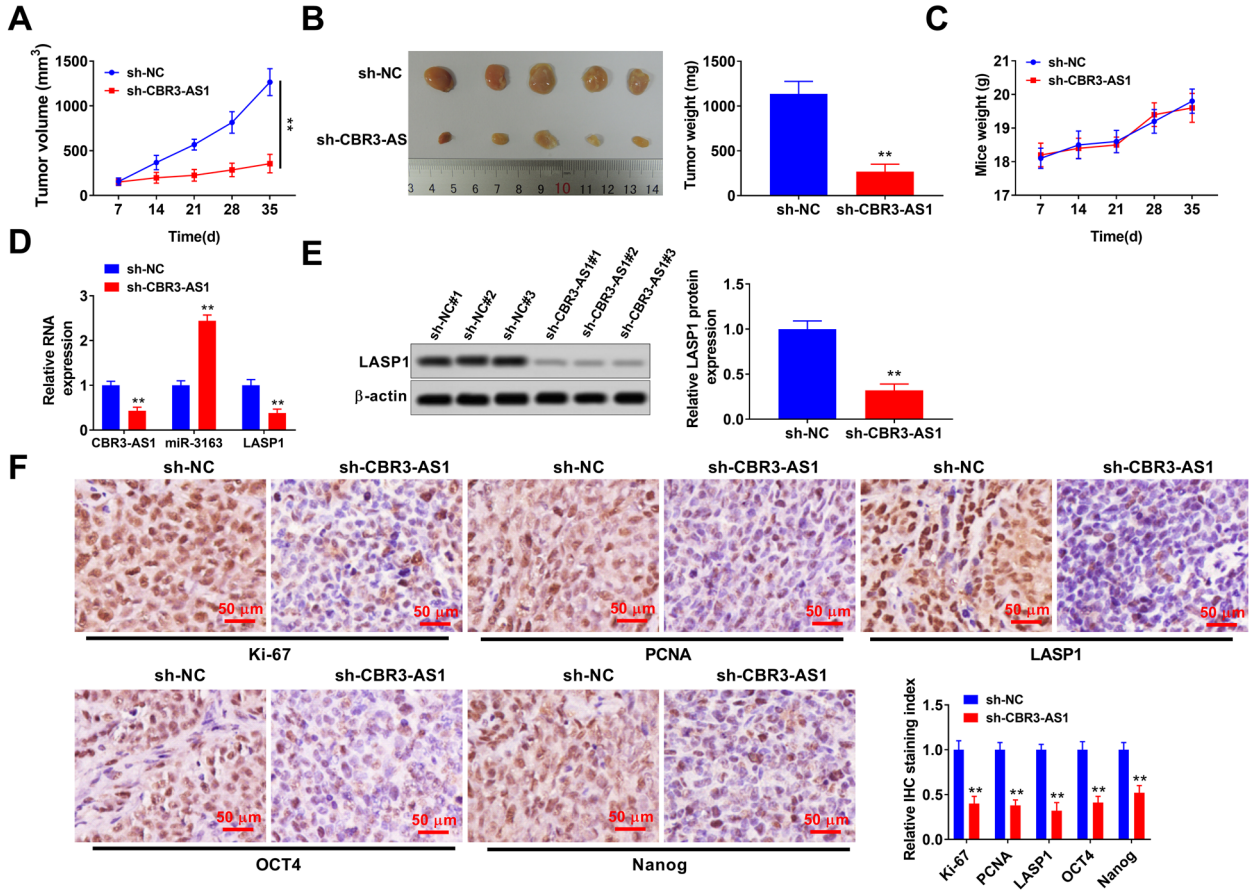

Figure 6. LncRNA CBR3-AS1 knockdown repressed cervical cancer growth and suppressed cancer stem cell-like properties *in vivo*. A–C) Mice were divided into the sh-CBR3-AS1 group and the sh-NC group. Tumor volume and weight in the two groups was measured on day 7, 14, 21, 28, and 35. D) CBR3-AS1, miR-3163, and LASP1 RNA expressions were measured using qRT-PCR. E) LASP1 expression was measured using western Blotting. F) Proliferation markers (Ki-67 and PCNA), LASP1, and stem cell markers (OCT4 and Nanog) expressions were determined by IHC staining. *p<0.05; **p<0.01

group than in the sh-NC group, whereas miR-3163 expression was significantly increased in the sh-CBR3-AS1 group than in the sh-NC group (Figure 6D). In addition, LASP1 protein expression was significantly downregulated in the sh-CBR3-AS1 group than in the sh-NC group (Figure 6E). IHC staining showed the expressions of proliferation markers (Ki-67 and PCNA), LASP1, and stem cell markers (OCT4 and Nanog) were inhibited in the sh-CBR3-AS1 group than in the sh-NC group (Figure 6F). These results indicated that lncRNA CBR3-AS1 facilitated tumor growth and cancer stem cell-like properties *in vivo*.

Discussion

LncRNA CBR3-AS1 is elevated in osteosarcoma, lung adenocarcinoma, and breast cancer, and the elevated expression of lncRNA CBR3-AS1 is related to a poor prognosis and a lower overall survival rate in these patients [11, 12, 24]. Besides, lncRNA CBR3-AS1 is elevated in cancer tissues and cancer cells which exerts an important function in promoting the growth, migration, metastasis of cancers [10]. LncRNA CBR3-AS1 knockdown repressed cell viability, invasion, and migration, whereas facilitated cell apoptosis in non-small cell lung cancer and breast cancer *in vitro* [11, 24]. Importantly, lncRNA CBR3-AS1 can regulate cancer stem cell-like properties to control the metastasis of cancer cells through interacting with Notch signaling [25]. However, the relationship between lncRNA CBR3-AS1 and the prognosis of cervical cancer patients is not elucidated, and the function of lncRNA CBR3-AS1 in cervical cancer progression is still not clear. So, it is of importance to discover the role of lncRNA CBR3-AS1 in cervical cancer cell proliferation and cancer stem cell-like properties in cervical cancer, as well as its relationship with the prognosis of cervical cancer patients. In this study, lncRNA CBR3-AS1 was elevated in cervical cancer tissues and cervical cancer cells. Patients with high expression of lncRNA CBR3-AS1 showed a lower overall survival rate, and high CBR3-AS1 expression was positively related to FIGO stage, distant metastasis, and lymphatic metastasis of cervical cancer. LncRNA CBR3-AS1 knockdown reduced cervical cancer cell proliferation and inhibited cancer stem cell-like properties. Therefore, lncRNA CBR3-AS1 functioned as an oncogene in cervical cancer and acted as an effective biomarker for the prognosis of cervical cancer.

Several studies have discovered that lncRNA CBR3-AS1 can suppress the expression of miRNAs by competitively binding with miRNAs in cancer cells [10, 26]. For example, lncRNA CBR3-AS1 can target to miR-25-3p as a competitive endogenous RNA (ceRNA) to activate the MAPK pathway, thereby modulating the drug sensitivity, viability, and apoptosis of breast cancer cells [26]. LncRNA CBR3-AS1 sponged miR-509-3p as a ceRNA to promote the expression of downstream target HDAC9, thereby driving the tumorigenicity of non-small-cell lung cancer [10]. In this study, luciferase reporter gene, RNA pull-down, and RIP assays were conducted to verify that lncRNA CBR3-AS1 could bind to miR-3163. In addition, lncRNA CBR3-AS1 knockdown reduced cervical cancer cell proliferation and inhibited cancer stem cell-like properties through upregulating miR-3163, indicating lncRNA CBR3-AS1/miR-3163 functioned a critical role in cervical cancer progression.

miR-3163 can be sponged by lncRNAs, such as lncRNA CTBP1-AS2, lncRNA OIP5-AS1, lncRNA MEG3, and lncRNA MAGI2-AS3, to exert important functions in the modulation of the progression of hepatocellular carcinoma, colorectal cancer, and non-small cell lung cancer [13, 14, 16, 27]. Importantly, miR-3163 can be targeted by lncRNA CTBP1-AS2 in cervical cancer cells, and miR-3163 acts as a vital molecule in the lncRNA CTBP1-AS2/miR-3163/ZNF217 signaling pathway to exert function in cervical cancer progression [13]. In addition, miR-3163 can significantly suppress many downstream target genes (VEGFA, ZNF217, TOP2A, and ADAM-17) in hepatocellular carcinoma, cervical cancer cells, and pancreatic cancer cells via binding to 3' UTRs of these targets, thereby to repress or facilitate cancer progression [13, 16]. In the current study, we identified that a ceRNA modulatory signaling pathway involving lncRNA CBR3-AS1 and miR-3163 in cervical cancer cells, and lncRNA CBR3-AS1 can negatively modulate miR-3163 expression in cervical cancer cells by binding to miR-3163.

LASP1 can be targeted by various miRNAs, such as miR-203a-3p, miR-3619, and miR-1294, to modulate the progression of retinoblastoma, esophageal cancer, and nasopharyngeal carcinoma [28–30]. LASP1 is highly expressed in cancer tissues and cancer cells and facilitates cancer cell proliferation, invasion, and metastasis, including breast cancer, thyroid cancer, pancreatic cancer, and colorectal cancer [31–35]. LASP1 expression can be repressed by the overexpressing of miR-1294, miR-218-5p, and miR-625-5p in cancer cells, which can reverse its function in cancer cells [28; 30; 31; 32; 33]. Regarding the regulation of stemness factors Nanog, OCT4, and SOX2 expressions by LASP1 [20], we considered that LASP1 can modulate cancer stem cell-like properties. In this study, mechanistic investigations showed LASP1 was targeted by miR-3163, and LASP1 expression was suppressed by miR-3163 in cervical cancer cells. Functionally, LASP1 played promoting effects on cervical cancer cells by promoting cell viability, proliferation, and cancer stem cell-like properties. Rescue experiments identified that decreasing miR-3163 or increasing LASP1 expression reversed CBR3-AS1 knockdown-induced suppressive effects on cervical cancer cells. Therefore, the lncRNA CBR3-AS1/miR-3163/LASP1 signaling pathway may exert a vital function in regulating the proliferation and cancer stem cell-like properties in cervical cancer.

According to the results of recent studies, lncRNA CBR3-AS1 has been verified to facilitate tumor growth and contribute to tumor progression *in vivo* xenograft mouse

model [11, 26]. Although studies have found that lncRNA CBR3-AS1 promotes the viability, proliferation, and migration of cancer cells, including osteosarcoma cells, breast cancer cells, and non-small cell lung cancer cells *in vitro* [12, 24, 26], the roles of lncRNA CBR3-AS1 in the tumor growth and cancer stem cell-like properties of cervical cancer cells *in vivo* are still not clear. In the current study, lncRNA CBR3-AS1 knockdown decreased tumor volume and weight in a HeLa xenograft mouse model. Moreover, lncRNA CBR3-AS1 knockdown suppressed proliferation markers (Ki-67 and PCNA), LASP1, and stem cell markers (OCT4 and Nanog) expressions, which identified that lncRNA CBR3-AS1 facilitated cervical cancer growth and cancer stem cell-like properties *in vivo*.

This study firstly identifies that lncRNA CBR3-AS1, miR-3163, and LASP1 can predict the prognosis of cervical cancer, and their correlation between clinicopathological parameter distant metastasis and lymphatic metastasis. In addition, the lncRNA CBR3-AS1/miR-3163/LASP1 signaling pathway exerts a vital function in the regulation of cervical cancer cell proliferation and cancer stem cell-like properties. However, the roles of the lncRNA CBR3-AS1/miR-3163/LASP1 signaling pathway in the modulation of invasion, metastasis, angiogenesis, and immune evasion in cervical cancer were not investigated, and we will focus on these problems in further studies.

In conclusion, we identified the lncRNA CBR3-AS1/miR-3163/LASP1 signaling pathway in the regulation of cervical cancer cell proliferation and cancer stem cell-like properties. Besides, high lncRNA CBR3-AS1 expression showed a lower overall survival rate, and the expressions of lncRNA CBR3-AS1, miR-3163, and LASP1 were closely related to distant metastasis and lymphatic metastasis of cervical cancer, which might provide molecular targets for repressing cervical cancer.

Supplementary information is available in the online version of the paper.

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https://doi.org/10.4149/neo_2022_220730N784

LncRNA CBR3-AS1 predicts a poor prognosis and promotes cervical cancer progression through the miR-3163/LASP1 pathway

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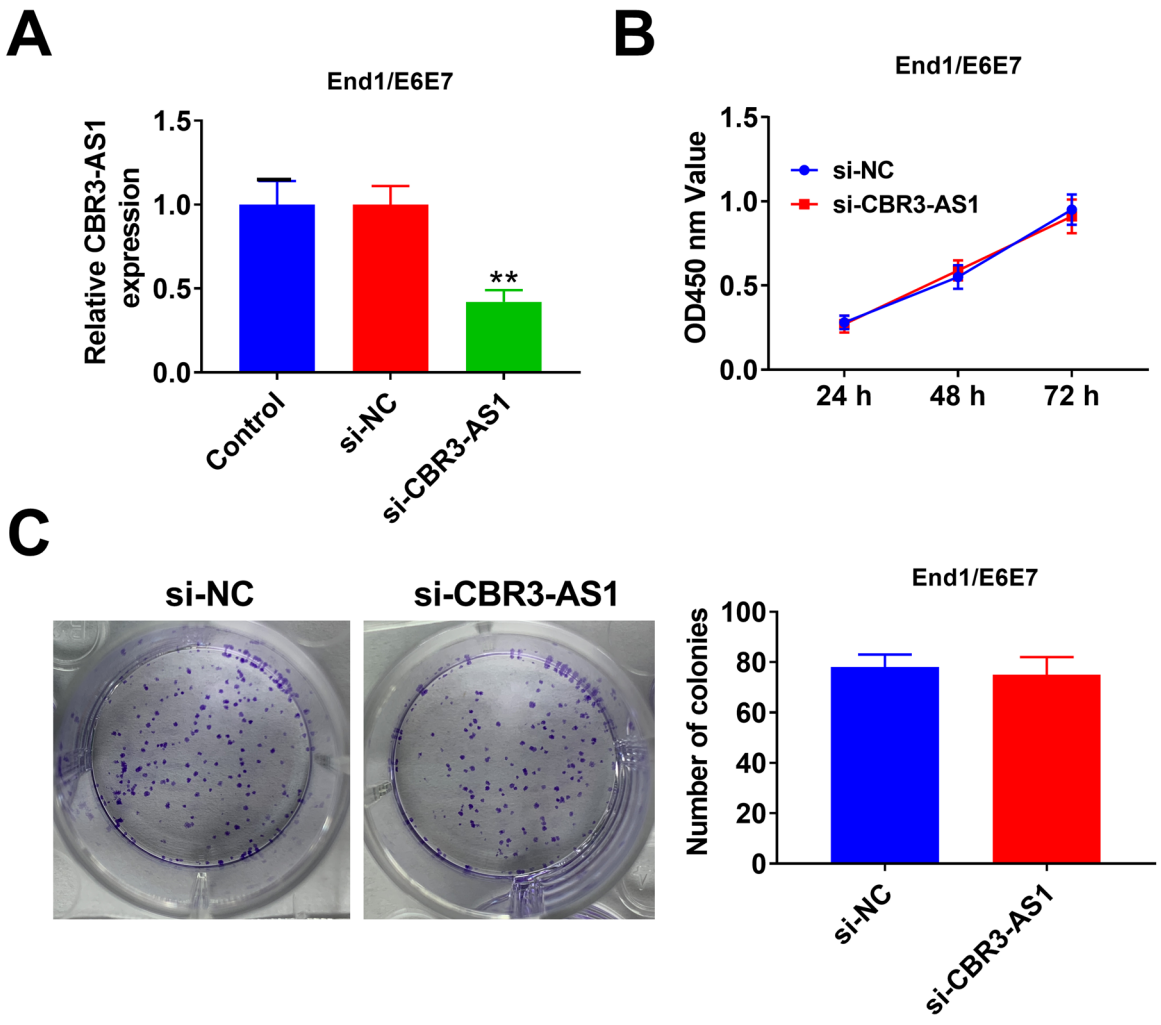
Supplementary Information



Supplementary Figure S1. The effects of CBR3-AS1 on colony formation and proliferation in End1/E6E7 cells. A) LncRNA CBR3-AS1 expression was measured using qRT-PCR in End1/E6E7 cells of control, si-NC, and si-CBR3-AS1 groups. B) Cell viability was measured using MTT assay. C. Colony formation ability of End1/E6E7 cells was measured using colony formation assay. **p < 0.01

Sequences of the CBR3-AS1-WT

GAAATAAATCCTCTTAAGGGGAGGAGGTGTCCTCTTTGATCTGAGGGCACACCTCTGGGAGAGAGGCCAGAGGCTGACAGTCAGCCTCGT-
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CAGAATCTGTACTTGGAGGCCCCATACAACCATTCTGCATTTCACTTTCAGTACAGATTCAATAAATTACATAAGATACTCAACATTTTATGAT

Sequences of the CBR3-AS1-MUT

GAAATAAATCCTCTTAAGGGGAGGAGGTGTCCTCTTTGATCTGAGGGCACACCTCTGGGAGAGAGGCCAGAGGCTGACAGTCAGCCTCGT-
CAGCCAGATGCGTCATCAGCCCTGGGAAGGGCTGCACCCACACCACCCTCCACTGAGGTCCAGGTGGCCCCACCGGTCCCGAAACACCTGCT-
GCCTTACTCTAGAAAGAGTAGGCAAGTTGCGCCCTCTAGTGGCTTAGTAGTTGCTTGTCCTATTGACTCAGAACCTTGAACAAATAAGCAAGT-
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Sequences of the LASP1 3'UTR-WT

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Sequences of the LASP1 3'UTR-MUT

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