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DOI: 10.14670/HH-18-527
Article type: ORIGINAL ARTICLE
Accepted: 2022-09-30
Epub ahead of print: 2022-09-30
The circCDK17/miR-122-5p/ASF1B axis regulates the progression of cervical cancer

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Abstract:

Background: Cervical cancer (CC) ranks fourth in terms of incidence and fourth in mortality overall in women worldwide. Circular RNAs (circRNAs) have been shown to be involved in the development of CC. However, the function of circRNA cyclin dependent kinase 17 (circCDK17, hsa_circ_0002762) in CC pathogenesis has not been studied.

Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of related genes. MTT, thymidine analog 5-ethynyl-2’-deoxyuridine (EdU), flow cytometry, transwell and wound-healing assays were designed to analyze cell proliferation, cell cycle progression, migration and invasion, respectively. Western blot was utilized to examine the protein levels of Cyclin D1, E-cadherin and Vimentin. The relationship between miR-122-5p and circCDK17 or ASF1B was verified by dual-luciferase reporter assay. The xenograft model was established to study the role of circCDK17 in vivo.

Results: CircCDK17 and anti-silencing function 1B histone chaperone (ASF1B) were highly expressed in CC tissues and cells. Silencing circCDK17 reduced the proliferation, migration and invasion of CC cells. MiR-122-5p was a target of circCDK17. Silencing circCDK17 inhibited the malignant behaviors of CC cells by releasing miR-122-5p. Moreover, ASF1B was a target of miR-122-5p. Overexpression of ASF1B partially restored the inhibitory effects of circCDK17 silencing on cell
proliferation, migration and invasion. Animal experiments confirmed the anti-tumor
effect of circCDK17 knockdown in vivo.

**Conclusion:** Our study demonstrates that circCDK17 regulates the expression of
ASF1B by miR-122-5p competition and thus promotes the development of CC,
providing a novel targeted therapy for CC.

**Keywords:** CircCDK17, miR-122-5p, ASF1B, cervical cancer

**Introduction**

Cervical cancer (CC) ranks fourth in terms of incidence and fourth in mortality
overall in women worldwide, threatening the life and health of women (Cohen et al.,
2019; Siegel et al., 2019). It is well known that about 95% of CC is caused by the
infection of the carcinogenic human papillomavirus (HPV) (Schiffman et al., 2011;
Scarth et al., 2021; Schmeler et al., 2022). According to the association with CC and
precursor lesions, HPVs can be grouped into low-risk (types 6, 11, 42, 43 and 44) and
high-risk (types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70) HPV
types (Burd 2003). Among them, HPV types 16 and 18 account for roughly 70% of all
cervical cancers (Crosbie et al., 2013). Although CC can be treated by surgical
removal of the uterus in the early stage (Biewenga et al., 2011; Fader 2018), patients
with advanced CC or invasive CC have a poor prognosis through chemotherapy and
radiotherapy (Jhamad et al., 2018). Therefore, it is particularly important to study the
molecular mechanism of CC development for developing effective molecular targets
for the treatment of CC.

Circular RNAs (circRNAs) are natural RNA circles with covalently closed continuous
structure, lacking 3’ and 5’ termini (Chen and Yang, 2015; Liu et al., 2017).
CircRNAs are involved in various cancer diseases, including CC (Chaichian et al.,
2020; Chen et al., 2021). Moreover, circRNAs can sponge microRNAs (miRNAs) and
thus affect gene expression at the post-transcriptional level (Hansen et al., 2013;
Salzman et al., 2013). For instance, Hu et al., established that circ_0067934 was
highly expressed in CC and it worked as a competing endogenous RNA (ceRNA) for
miR-545 to contribute to the development of CC by upregulating EIF3C (Hu et al.,
2019). Chen et al., uncovered that circ_0084927 exerted a strong oncogenic role in
CC by inducing ARL2 expression through miR-142-3p competition and was present
at high levels in CC (Chen et al., 2020). Conversely, several circRNAs, such as
circ_0107593 and circ_VPRBP, are present with low expression in CC and can function as tumor suppressors in CC through their ceRNA activity (Liao et al., 2020; Shen et al., 2022). By analyzing the microarray expression profile (GSE102686) in CC, we found that circRNA cyclin dependent kinase 17 (circCDK17, hsa_circ_0002762) was highly expressed in CC, consistent with a previous report (Huang et al., 2021). Moreover, inhibition of circCDK17 can diminish CC malignant progression through miR-1294-dependent modulation of YWHAZ (Chen et al., 2022), suggesting the oncogenic role of circCDK7 in CC. Nonetheless, our understanding of the precise action played by circCDK17 in CC pathogenesis is still limited.

A large number of documents have reported the dual regulatory role of miRNAs in the occurrence and progression of CC by working as tumor drivers or anti-tumor factors (Morgan et al., 2020; Hoelzle et al., 2021; Martínez-Noël et al., 2021). Examples of anti-tumor miRNAs in CC include miR-34a-5p, which is underexpressed in CC and diminishes CDC25A expression, and miR-186-5p, which targets and inhibits FZD3 and is present at low levels in CC (Jiang et al., 2021; Wang et al., 2021). Conversely, the oncogenic miR-9-5p and miR-1908 are highly expressed in CC and control the expression of their targets (Wei et al., 2019; Yu et al., 2021). As for miR-122-5p, it has been found to play an anti-tumor role in CC (Ding et al., 2019; Li et al., 2019; Gao et al., 2021).

Anti-silencing function 1 (ASF1), the histone chaperone protein, has two existing forms, ASF1A and ASF1B (Peng et al., 2010; Paul et al., 2016). Studies have shown that ASF1B is actively involved in the development of various cancers, such as breast cancer and prostate cancer (Corpet et al., 2011; Han et al., 2018; Liu et al., 2020). Importantly, in CC, ASF1B has been demonstrated to function as a strong driver by regulating CDK9 stabilization (Liu et al., 2020).

In a preliminary survey for the molecular basis of circCDK17 using target prediction programs (circBank (Liu et al., 2019) and starBase (Li et al., 2014), we observed a putative relationship between miR-122-5p and circCDK17 or ASF1B, suggesting the implication of the circCDK17/miR-122-5p/ASF1B axis in CC. We therefore explored whether the putative ceRNA cascade is causally involved in CC pathogenesis. Our current study can provide a novel molecular explanation for the oncogenic activity of circCDK17 in CC pathogenesis.
Materials and methods

Clinical tissue samples

The study was approved by Guizhou Provincial People's Hospital Ethics Committee. 55 pairs of fresh cancer and paracancerous cervical tissue samples were harvested from 55 patients with CC at Guizhou Provincial People's Hospital. All patients gave written informed consent. The clinicopathologic features of these patients are shown in Table 1. Subsequently, the samples were frozen and preserved at -80°C for use.

Cell lines and cell culture

Human normal cervical epithelial cells (HUCECs) and CC cell lines (Hela, SiHa, CaSkI and C33A) were purchased from American type culture specimens (ATCC; Manassas, VA, USA). All CC cells were cultured in Dulbecco’s modified eagle medium (DMEM; Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin (Invitrogen, Carsbad, CA, USA) at 37°C in 5% CO₂ environment. HUCECs were cultured using standard protocols provided by ATCC.

Cell transfection

Plasmids expressing short hairpin RNA (shRNA)-circCDK17 (sh-circCDK17, 5’-UGUGCGUUGAAAAAUCCUAUU-3’) and its corresponding control plasmid (sh-NC, 5’-UCUCGGUAGUAUAUUGCAAUU-3’) were purchased from Ribobio (Guangzhou, China). Mature miR-122-5p mimic, mimic negative control (miR-NC), miR-122-5p inhibitor (in-miR-122-5p, the exact antisense of the mature miR-122-5p sequence, 5’-ACCUCACACUGUACCACAAAC-3’), and negative control inhibitor (in-miR-NC) were obtained from Ribobio. pcDNA3.1(+) -based ASF1B overexpression vector (expressing the coding sequence without 3’UTR) pcDNA-ASF1B and a nontarget negative control plasmid (pcDNA) were synthesized by Ribobio. All transient transfections were performed using Lipofectamine 2000 (Thermo Fisher) as per the accompanying protocols. Briefly, Hela and SiHa CC cells were plated in 12-well culture dishes at 2 × 10⁵ cells per well 24 h before transfection. Next day, a mixture of 300 ng plasmid or/and 50 nM miRNA mimic or/and 50 nM miRNA inhibitor and Lipofectamine 2000 was prepared and then dispensed into each well. After 8 h incubation, media were replaced by fresh complete growth medium.
Transfected cells were harvested for in vitro studies after culturing for 24 h.

**RNA preparation, RNase R treatment and quantitative real-time polymerase chain reaction (qRT-PCR)**

The Trizol Reagent (Thermo Fisher) was used to separate total RNA from collected tissues and cultured cells as per the manufacturing instructions. RNase R treatment was done by adding 3 U RNase R (Geneseed, Guangzhou, China) or mock control into 1 μg total RNA and 10 min incubation at 37°C. For circCDK17 and mRNA analyses, total RNA (1 μg) was reverse-transcribed using RevertAid Reverse Transcription Kit (Thermo Fisher) with random hexamers. Then, qRT-PCR was performed in a final volume of 25 μL containing 5 μL of diluted cDNA, 12.5 μL of SYBR Green qRT-PCR Mix (Takara, Shiga, Japan), 1.5 μL of designed primers (10 μM) and 6 μL of H2O. For miRNA analysis, cDNA was synthesized from 100 ng total RNA using BON miRNA 1st-strand cDNA Synthesis Kit (Bonyakhteh, Tehran, Iran) and then amplified by qRT-PCR using SYBR Green qRT-PCR Mix with primers specific to miRNAs. Samples were amplified for 40 cycles using the following parameters: 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute. GAPDH (for circCDK17 and mRNAs) or U6 (for miRNAs) were used as an internal reference, and the relative expression was calculated by the method 2-ΔΔCT. A list of primer sequences is available in Supplement Table 1.

**Western blot analysis**

Total protein from collected tissues and cultured cells was prepared using Radioimmunoprecipitation lysis buffer (RIPA; Thermo Fisher) containing a protease inhibitor cocktail (Thermo Fisher). The BCA protein assay kit (Pierce; Rockford, IL, USA) was used to quantify the concentration of total protein. Protein (30 μg/lane) was resolved on the 4-20% SDS-polyacrylamide gradient gels (Bio-Rad, Marnes-la-Coquette, France), and the resulting gels were blotted to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After being blocked with 5% skim milk, the membranes were probed with primary antibodies including anti-β-actin (1:1,000, ab8226, Abcam, Cambridge, MA, USA), anti-Cyclin D1 (1:200, ab16663, Abcam), anti-Epithelial cadherin (E-cad; 1:1,000, ab231303, Abcam), anti-Vimentin (1:1,000, ab20346, Abcam), and anti-ASF1B (1:1,000, ab235358, Abcam) antibodies overnight at 4°C. The PVDF membranes were washed 3 times with 1X TBST buffer.
and then incubated with goat anti-rabbit or anti-mouse IgG secondary antibodies conjugated with HRP (1:5,000, ab205718 or ab205719, Abcam) for 1 h at room temperature. Signals were visualized using BeyoECL Plus kit (BeyoTime, Shanghai, China), and the densitometry of band intensity was analyzed using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide (MTT) cell proliferation assay
Transfected Hela and SiHa cells were incubated in 96-well plates for 0, 24, 48, or 72 h, and then 10 µL MTT (Sigma, St Louis, MO, USA) was added to each well for 4 h. 100 µL dimethyl sulfoxide (DMSO; Sigma) was added to dissolve crystal violet precipitates and incubation was done for 10 min. Finally, we measured the absorbance at 490 nm.

5-ethynyl-2′-deoxyuridine (EdU) assay
Transfected Hela and SiHa cells (2×10^3 cells/mL) were seeded into 96-well dishes in 200 µL medium supplemented with 10% FBS 24 h before treatment with 50 µM of EdU reagent according to the Cell-Light EdU DNA Cell Proliferation Kit (Ribobio) at 37°C for 2 h. The EdU staining was performed using 1× Apollo488 for 30 min, and the cell nuclei were stained with DAPI for 30 min. The images were obtained using a fluorescence microscope (Leica, Wetzlar, Germany).

Flow cytometry
Flow cytometry (BD Biosciences, San Diego, CA, USA) was used to detect cell cycle. Briefly, transfected Hela and SiHa cells were incubated with 1 mL of PI/TritonX-100 staining solution (containing 0.2 mg RNase A, 20 µg of PI, and 0.1% TritonX-100) at 4°C for 30 min, and data were analyzed within 1 h.

Cell migration and invasion assays
Transwell invasion and wound-healing assays were manipulated to assess cell invasion and migration, respectively. For transwell invasion assay, the Transwell insert chambers (BD Biosciences) were utilized. 5×10^4 transfected Hela and SiHa cells were added into the upper of Transwell chambers. Meanwhile, the Transwell lower chambers were added with fresh culture medium containing 10% FBS. The
number of invaded cells was counted after 24 h under a high-powered microscope.

For wound-healing analysis, cells were seeded into 6-well plates, and scratches were then made using pipette tips (200 µL) after 24 h. Serum-free medium was added and cultured for 24 h. Photographs were taken for observation.

**Dual-luciferase reporter assay**

The segments of circCDK17 and ASF1B 3’UTR containing the presumed wild-type (WT) miR-122-5p interacting sites (UCACACUCC in circCDK17, CACUCC in ASF1B 3’UTR) or mutant-type (MUT) target sequence (AGUGUGAGG in circCDK17, GUGAGG in ASF1B 3’UTR), synthesized by Genscript (Nanjing, China), were cloned into the Sac I and Pme I sites of the pMIR-REPORT vectors (Realgene, Nanjing, China), respectively. Hela and SiHa cells in 24-well plates (5×10^5 cells/well) were transfected with luciferase reporter constructs (100 ng), pRL-TK Renilla control vector (50 ng, Promega, Tokyo, Japan) in combination with miR-122-5p mimic (10 nM) or miR-NC mimic by Lipofectamine 2000 (Thermo Fisher). The relative luciferase activities were measured by Dual-Luciferase Reporter Assay Kit (GeneCopoeia, Rockville, MD, USA) after 48 h transfection, with Renilla luciferase activity as the control.

**RNA pull-down assay**

Lysates of Hela and SiHa cells extracted with RIPA buffer were incubated with the biotinylated circCDK17 probe and negative control Oligo probe (both from Ribobio) and streptavidin magnetic beads (Thermo Fisher) overnight at 4°C. RNA was prepared from the beads and measured by qRT-PCR for evaluation of miRNA expression.

**Immunohistochemistry (IHC)**

Xenograft tissues were fixed with 4% buffered paraformaldehyde, dehydrated and embedded in paraffin. Paraffin sections (5 µm) were dewaxed and rehydrated for antigen stripping. The membranes were incubated with anti-Ki-67 (1:2,00, ab15580; Abcam) or anti-ASF1B (1:100, ab235358, Abcam) antibody at 4°C overnight, then incubated with Goat against mouse IgG secondar antibody (1:10,000, ab205719, Abcam) at room temperature for 1 h. Sections were stained with diaminobenzidine (DAB) kit (Sigma) according to the protocols. The positive staining cells were
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observed with a light microscope.

Xenograft models

All animal experiments were carried out in accordance with the instructions of Animal Care and Use Committee of Guizhou Provincial People's Hospital. Experimental mice (BALB/c, female, 6-8 weeks of age) were purchased from Beijing Weidahe Laboratory Animal Science and Technology Co., Ltd. (Beijing, China). Sh-circCDK17 lentivirus and its negative control (sh-NC) were obtained from Ribobio. Mice were injected with stably expressed sh-circCDK17 or sh-NC Hela cells at $1 \times 10^6$ cells/mouse, and the subcutaneous tumor size was measured on days 7, 14, 21 and 28 after cell injection. Each group included 6 mice. Tumor volume was calculated according to the formula: volume = $1/2 \times (\text{length} \times \text{width}^2)$. All mice were sacrificed and the tumors were measured. The expression of circCDK17 and miR-122-5p were analyzed by qRT-PCR, and the protein expression of ASF1B and Ki67 was analyzed by IHC.

Statistical analysis

GraphPad Prism 7 (GraphPad, San Diego, CA, USA) was utilized for data analysis. Difference comparison between groups was determined using Student’s $t$-test or ANOVA with post hoc Tukey’s multiple comparisons test. Pearson’s correlation analysis was applied to measure the expression correlation. Each experiment was carried out at least three times and the data were shown as the mean ± standard deviation (SD). $P < 0.05$ meant significant difference.

Results

CircCDK17 was highly expressed in CC tissues and cells

Using circRNA microarray analysis, the GSE102686 database showed that circCDK17 (hsa_circ_0002762) was highly expressed in cervical squamous cell carcinoma (SCC) (Figure 1A). CircCDK17, a 312 bp long circRNA, is derived from the CDK17 gene located on chromosome 12 and is formed by back-splicing of exon 2 and 3 of CDK17 pre-mRNA (Figure 1B). The high expression of circCDK17 in 5 cervical SCC tissues was also confirmed by qRT-PCR in the GSE102686 database (Figure 1C). In this study, we first confirmed the overexpression of circCDK17 in a cohort of 55 primary cervical tumor samples. qRT-PCR showed that circCDK17 was
upregulated in CC tissues compared with adjacent normal tissues (n = 55) (Figure 1D). Moreover, circCDK17 was present at high levels in CC cells compared with normal cervical epithelial HUCECs (Figure 1E). Furthermore, RNase R treatment results showed that circCDK17, but not the linear CDK17 mRNA, was resistant to RNase R (Figure 1F and 1G), indicating that circCDK17 has a higher tolerance to exonucleases. Additionally, circCDK17 expression was closely correlated with the TNM grade, tumor size, lymph node metastasis, and HPV status of the tumors (Table 1).

**CircCDK17 silencing inhibited the proliferation, migration and invasion of CC cells**

Next, we elucidated the functional role of circCDK17 in CC progression by knocking down circCDK17. The transfection efficiency of sh-circCDK17 in silencing circCDK7 was detected by qRT-PCR (Figure 2A). Strikingly, sh-circCDK17 transfection notably decreased cell viability and proliferation of Hela and SiHa cells (Figure 2B-2D). Flow cytometry results revealed that the number of cells in S stage was reduced by circCDK17 silencing, indicating that circCDK17 silencing suppressed cell cycle progression (Figure 2E and 2F). Moreover, silencing of circCDK17 reduced cell migration and invasion abilities of Hela and SiHa cells (Figure 2G and 2H). Additionally, western blot results showed that circCDK17 silencing remarkably decreased the levels of the cell cycle marker Cyclin D1 and migration-related protein Vimentin and increased the expression of E-cad in the two cell lines (Figure 2I and 2J). Taken together, knockdown of circCDK17 inhibited CC cell proliferation, migration and invasion.

**MiR-122-5p was a target of circCDK17**

To identify the mechanism underlying the regulation of circCDK17 in CC progression, we used two prediction programs starBase (http://starbase.sysu.edu.cn/) and circBank (http://www.circbank.cn/) to predict the putative targeted miRNAs of circCDK17. As shown in Figure 3A, Venn diagram showed six miRNAs that overlapped among the two algorithms. To verify the direct relationship between the six miRNAs and circCDK17, we adopted RNA pull-down assays. Among these candidates, miR-122-5p was preferentially enriched by biotinylated circCDK7 probe (Figure 3B and 3C). Herein, we selected miR-122-5p for further investigation. Figure
3D shows the putative binding sites between circCDK17 and miR-122-5p. To validate this, we generated wild-type (WT) or mutant-type (MUT) circCDK17 reporter constructs and transfected them into cells along with miR-122-5p mimic. The overexpression efficiency of miR-122-5p mimic was confirmed by qRT-PCR (Figure 3E). Dual-luciferase reporter results showed that overexpression of miR-122-5p reduced the luciferase activity of circCDK17 WT, rather than circCDK17 MUT (Figure 3F and 3G). Additionally, the expression of miR-122-5p was decreased in CC tissues (n = 55) compared with adjacent normal cervical tissues (n = 55) (Figure 3H). Pearson’s correlation analysis showed that miR-122-5p expression was negatively correlated with circCDK17 level in CC tissues (Figure 3I). Moreover, qRT-PCR showed the downregulation of miR-122-5p in Hela and SiHa cells compared with normal cervical epithelial HUCECs (Figure 3J). Hence, circCDK17 targeted miR-122-5p by binding to miR-122-5p.

The effects of circCDK17 silencing on cell proliferation, migration and invasion were reversed by miR-122-5p reduction
The aforementioned findings prompted us to examine whether the effects of circCDK17 silencing are due to the upregulation of miR-122-5p. To resolve this, we reduced miR-122-5p with miR-122-5p inhibitor (in-miR-122-5p) in circCDK17-silenced cells. QRT-PCR confirmed the miR-122-5p reduction efficacy of in-miR-122-5p in Hela and SiHa cells (Figure 4A). Remarkably, miR-122-5p reduction rescued circCDK17 knockdown-driven cell viability and proliferation defects (Figure 4B-4D). Moreover, transfection of in-miR-122-5p reversed circCDK17 silencing-mediated cell cycle arrest (Figure 4E and 4F). Also, silencing circCDK17 reduced cell migration and invasion, while these effects were partially reversed by miR-122-5p reduction (Figure 4G and 4H). Western blot results revealed that miR-122-5p reduction abolished the impact of sh-circCDK17 on Cyclin D1, Vimentin and E-cad expression in Hela and SiHa cells (Figure 4I and 4J). Together, these data supported our hypothesis that the effects of circCDK17 silencing are partially due to the upregulation of miR-122-5p.
ASF1B was a target of miR-122-5p and was regulated by circCDK7 through miR-122-5p

To elucidate how miR-122-5p influences CC development, we considered the upregulated genes in CC to be potential targets of miR-122-5p. Using starBase software, we selected some genes that were upregulated in CC and found that ASF1B mRNA expression was the most significantly reduced in miR-122-5p-expressing Hela and SiHa cells (Figure 5A and 5B). We thus focused on ASF1B in this study. As shown in Figure 5C, starBase software showed the predicted binding sites between miR-122-5p and ASF1B 3’UTR. The results of dual-luciferase reporter assays showed that miR-122-5p overexpression repressed the luciferase activity of ASF1B 3’UTR-WT, but not ASF1B 3’UTR-MUT (Figure 5D and 5E). Western blot analysis showed that ASF1B protein was significantly increased in Hela and SiHa CC cells compared with normal HUCECs (Figure 5F). Moreover, in Hela and SiHa CC cells, circCDK17 knockdown led to a distinct downregulation in ASF1B protein expression, and this effect was strongly abolished by miR-122-5p inhibitor (Figure 5G). Additionally, qRT-PCR results showed that ASF1B mRNA expression was increased in CC tissues (n = 55) compared with paired normal tissues (n = 55) (Figure 5H). Consistently, western blot results confirmed the high expression of ASF1B protein in CC tissues (n = 3) compared with paired normal tissues (n = 3) (Figure 5I). Expression correlation analyses using Pearson’s correlation analysis showed that ASF1B expression was negatively correlated with miR-122-5p level and positively correlated with circCDK17 expression (Figure 5J and 5K). These data indicated that circCDK17 regulated ASF1B expression through miR-122-5p.

The effects of circCDK17 silencing on cell proliferation, migration and invasion were reversed by overexpression of ASF1B

Based on the repression of circCDK17 knockdown in ASF1B expression, we decided to explore whether ASF1B is a downstream effector of circCDK17 in regulating CC progression. Western blot confirmed the ASF1B overexpression efficiency of ASF1B expression plasmid in Hela and SiHa cells (Figure 6A). Indeed, increased expression of ASF1B lacking 3’UTR, and therefore insensitive to miR-122-5p, restored sh-circCDK17-imposed cell viability and proliferation reduction (Figure 6B-6D). Overexpression of ASF1B also reversed circCDK17 silencing-driven cell cycle arrest in Hela and SiHa cells (Figure 6E and 6F). Moreover, ASF1B restoration abated
circCDK17 silencing-driven cell migration and invasion defects (Figure 6G and 6H). Additionally, sh-circCDK17 transfection remarkably decreased the levels of Cyclin D1 and Vimentin and increased the expression of E-cad, while these effects were partially reversed by overexpression of ASF1B (Figure 6I and 6J). Hence, ASF1B seemed to be a functionally downstream effector of circCDK17.

**CircCDK17 knockdown inhibited tumor growth in vivo**

In order to explore the influence of circCDK17 in tumorigenicity, we constructed xenograft mice models in vivo. Transduction of sh-circCDK17 lentivirus significantly inhibited tumor growth, as presented by the reduced tumor volume (Figure 7A) and tumor weight (Figure 7B). Then, qRT-PCR was used to analyze the expression of circCDK17 and miR-122-5p, and the results showed that transduction of sh-circCDK17 lentivirus reduced the expression of circCDK17 and enhanced the expression of miR-122-5p in the xenograft tumors (Figure 7C and 7D). Furthermore, IHC results showed that the sh-circCDK17-transduced tumors had fewer cells stained with ASF1B and the proliferating marker Ki67 than the controls (Figure 7E). In conclusion, down-regulation of circCDK17 inhibited tumor growth in vivo.

**Discussion**

The importance of circRNAs in CC pathogenesis has become increasingly clear in recent years (Chaichian et al., 2020; Chen et al., 2021). Intense studies have highlighted that circRNA-mediated ceRNA crosstalk through miRNAs has been implicated in the pathogenesis of human cancers, including CC (Luo et al., 2021). Here, we confirmed the oncogenic activity of circCDK17 in CC, in agreement with a recent document (Chen et al., 2022). Importantly, we unveiled a novel mechanism, wherein circCDK17 post-transcriptionally modulates ASF1B expression by miR-122-5p competition, thereby contributing to CC development. Consistent with the findings reported by Chen et al. (Chen et al., 2022) and the GSE102686 dataset, our results validated that circCDK17 was up-regulated in human CC, suggesting its potential value as a diagnostic marker in CC. By using the shRNA silencing experiments, we demonstrated the oncogenic role of circCDK17 in CC partially by promoting cell growth and metastasis (Chen et al., 2022). As described for other circRNAs (Peng et al., 2020; Guo et al., 2021), circCDK17 is a true circular transcript and has a higher tolerance to exonucleases because of its RNase R
Further, we first demonstrated that miR-122-5p was a target of circCDK17. Numerous studies have documented the anti-tumor activity of miR-122-5p in human cancers. For instance, miR-122-5p inhibits the proliferation of gastric cancer cells and impedes the progression of gastric cancer (Pei et al., 2017; Xu et al., 2018). Moreover, miR-122-5p is down-regulated in CC and possesses a potent tumor-suppressive role (Ding et al., 2019; Li et al., 2019). We also tied in the regulation of circCDK17 in CC progression through miR-122-5p. Similarly, MIR205HG, a highly expressed long noncoding RNA (lncRNA) in CC, has been reported to promote CC progression depending on the modulation of miR-122-5p (Li et al., 2019).

Furthermore, we first identified ASF1B as a direct and functional target of miR-122-5p. ASF1B is crucial for the development of various cancers, such as lung adenocarcinoma and hepatocellular carcinoma (Ouyang et al., 2021; Zhang et al., 2021). ASF1B has also been established as a functionally downstream effector of miR-214-3p in the context of repressing multiple myeloma cell growth (Zhang et al., 2020). Of note, ASF1B has been discovered to function as a strong driver in CC by regulating CDK9 stabilization (Liu et al., 2020). More importantly, we first pointed to the ceRNA activity of circCDK17 to regulate ASF1B expression through shared miR-122-5p, indicating the circCDK17/miR-122-5p/ASF1B ceRNA crosstalk in CC.

Similarly, Chen et al., unveiled that circCDK17 operated as a ceRNA to promote cervical tumorigenesis through the miR-1294/YWHAZ regulation cascade (Chen et al., 2022). These findings suggest that the circCDK17/miR-122-5p/ASF1B and circCDK17/miR-1294/YWHAZ ceRNA networks may be two interactional or paralleled networks in regulating CC development. One circRNA can sponge many miRNAs, and one miRNA can target many genes. There may be other ceRNA mechanisms that remain to be defined in the regulation of circCDK17.

However, the \textit{in vivo} direct evidence between tumor growth suppression and the circCDK17/miR-122-5p/ASF1B ceRNA network is limited, which is expected to be studied in further work. A future challenge will be to determine how the novel ceRNA network regulates CC development \textit{in vitro} and \textit{in vivo}. Additionally, miR-122-5p can repress ovarian cancer cell metastasis by regulating E-cad and Vimentin (Duan et al., 2018). ASF1B is reported to contribute to human tumorigenesis by promoting cancer cell proliferation by upregulating the cell cycle marker cyclin D1 (Han et al., 2018; Wang et al., 2022). Our findings established the notion that the regulation of the
circCDK17/miR-122-5p/ASF1B ceRNA crosstalk in CC cell proliferation and invasion is at least in part due to the modulation of cyclin D1, E-cad, and Vimentin. Based on the observations in this study, inhibiting circCDK17 may represent a potential anti-tumor strategy in CC that strongly diminishes the malignant phenotypes of CC cells. We envision that the circCDK17 shRNA may be a starting point for the development of circRNA-based molecular therapies against CC. Future work is required to elucidate the long-term efficacy and safety of such agents in multiple experimental models.

Collectively, we establish herein circCDK17 as an oncogenic regulator of CC progression. We uncover a novel circCDK17/miR-122-5p/ASF1B ceRNA network in CC development. Since circCDK17 depletion suppresses tumor growth, the circCDK17 inhibitors appear to be promising candidates for the development of new anti-tumor therapies.

Acknowledgment
None

Disclosure of interest
The authors declare that they have no conflicts of interest

Funding
None

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Liao W., He J., Disoma C., Hu Y., Li J., Chen G., Sheng Y., Cai X., Li C., Cheng K.,


Figure legends

**Figure 1. CircCDK17 was highly expressed in CC tissues and cells.** (A) CircRNA expression profile of the GSE102686 database. (B) The structure of circCDK17. (C-E) The expression of circCDK17 was detected by qRT-PCR in cervical squamous cell carcinoma tissues (n = 5) and paired-paracancerous cervical tissues (n = 5) (C), CC tissues (n = 55) and adjacent normal cervical tissues (n = 55) (D), CC cells and normal cervical epithelial cells (E). (F and G) Effect of RNase R treatment on circCDK17 and the linear CDK17 mRNA in Hela and SiHa cells. **P < 0.01, ***P < 0.001.

**Figure 2. CircCDK17 silencing inhibited the malignant behaviors of CC cells.** (A-J) Hela and SiHa cells were transfected with sh-circCDK7 or sh-NC vector. (A) The expression of circCDK17 was detected by qRT-PCR. (B, C) MTT was used to detect cell viability. (D) Cell proliferation was measured by EdU assay. (E, F) The cell cycle was detected by flow cytometry. (G) Wound healing assay was used to detect cell migration. (H) Transwell assay detected cell invasion. (I, J) Western blot measured the expression of relative proteins. **P < 0.01.
Figure 3. MiR-122-5p was a target of circCDK17. (A) Using starbase and circbank programs to predict the miRNAs combined with circCDK17. (B, C) The relative miRNA enrichment was detected by RNA pull-down assay. (D) The binding sites between circCDK17 and miR-122-5p were analyzed using starbase. (E) QRT-PCR detected the expression of miR-122-5p. (F, G) Dual-luciferase reporter assays were performed to confirm the association between circCDK17 and miR-122-5p. (H) QRT-PCR detected the expression of miR-122-5p in CC tissues (n = 55) and adjacent normal cervical tissues (n = 55). (I) Pearson’s correlation analysis for the expression correlation between circCDK7 and miR-122-5p. (J) QRT-PCR detected the expression of miR-122-5p in CC cells and normal cervical epithelial cells. ***P < 0.001.

Figure 4. The effects of sh-circCDK17 on cell behaviors were eliminated by miR-122-5p inhibitor in CC cells. (A) The expression of miR-122-5p was detected by qRT-PCR in cells transfected with in-miR-122-5p or in-miR-NC. (B-J) Hela and SiHa cells were transfected with sh-NC+in-miR-NC, sh-circCDK7+in-miR-NC or sh-circCDK7+in-miR-122-5p. (B, C) MTT was used to detect cell viability. (D) Cell proliferation was measured by EdU assay. (E, F) The cell cycle was detected by flow cytometry. (G) Wound healing assay was used to detect cell migration. (H) Transwell assay detected cell invasion. (I, J) Western blot measured the expression of relative proteins. *P < 0.05, **P < 0.01.

Figure 5. ASF1B was the direct target of miR-122-5p. (A, B) Expression of mRNAs were detected by qRT-PCR in Hela and Siha cells transfected with miR-122-5p mimic or miR-NC mimic. (C) The complementary sequences between miR-122-5p and ASF1B were shown. (D, E) Dual-luciferase reporter assays were performed to confirm the association between miR-122-5p and ASF1B. (F, G) Western blot detected the expression of ASF1B in cells as indicated. (H) QRT-PCR detected the expression of ASF1B mRNA in CC tissues (n = 55) and adjacent normal cervical tissues (n = 55). (I) Western blot detected the expression of ASF1B protein in CC tissues (n = 3) and adjacent normal cervical tissues (n = 3). (J, K) Pearson’s correlation analysis. **P < 0.01, ***P < 0.001.
Figure 6. The effects of sh-circCDK17 on cell behavior were eliminated by ASF1B overexpression in CC cells. (A) The expression of ASF1B was detected by western blot in cells after transfection by ASF1B expression plasmid or pcDNA. (B-J) Hela and SiHa cells were transfected with sh-NC+pcDNA, sh-circCDK7+pcDNA or sh-circCDK7+ASF1B expression plasmid. (B, C) MTT was used to detect cell viability. (D) Cell proliferation was measured by EdU assay. (E, F) Cell cycle was detected by flow cytometry. (G) Wound healing assay was used to detect cell migration. (H) Transwell assay detected cell invasion. (I, J) Western blot measured the expression of relative proteins. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7. CircCDK17 knockdown inhibited tumor growth in vivo. (A, B) Tumor volume, representative images and weight of the xenograft tumors in vivo. (C, D) Relative expression levels of circCDK17 and miR-122-5p in xenografts were detected by qRT-PCR. (E) The positive rate of ASF1B and Ki67 was analyzed by IHC. ***P < 0.001.
### Table 1. The association between circCDK17 expression and the clinicopathologic features of cervical cancer patients (n=55)

<table>
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<tr>
<th>Characteristics</th>
<th>Number</th>
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<tr>
<td></td>
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<td>Low (n=27)</td>
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<td>Age (years)</td>
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<tr>
<td>≤60</td>
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<td>12</td>
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<tr>
<td>&gt;60</td>
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<td></td>
</tr>
<tr>
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<tr>
<td>III</td>
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TNM: tumor node metastasis. *P < 0.05 by chi-square test.
### Supplementary Table S1. Primer sequences for qRT-PCR

<table>
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<tr>
<th>Genes</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
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<td>CAGTCGCTTGTCGTGGAGT</td>
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<td>ASF1B</td>
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<td>ACCAGCACCAGTCTAGGAT</td>
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<td>miR-200a-3p</td>
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<td>miR-766-5p</td>
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<td>miR-141-3p</td>
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<td>miR-4677-3p</td>
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<td>CAGTCGCTTGTCGTGGAGT</td>
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<td>miR-500b-5p</td>
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<td>CAGTCGCTTGTCGTGGAGT</td>
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<td>CTCGCTTCCGCGACATACAT</td>
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**A**

GSE102686: SCC vs Normal

![Volcano plot showing expression changes](image)

**B**

Chr12(q23.1)

![Gene expression and back-splicing](image)

**C**

**GSE102686**

<table>
<thead>
<tr>
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<th>Relative expression of circCDK17</th>
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<tr>
<td>Normal</td>
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**D**

Relative expression of circCDK17

![Expression levels in CC and Normal](image)

**E**

Relative expression of circCDK17

![Expression levels across cell lines](image)

**F**

Relative RNA expression

![Expression levels in Mock and RNase R-treated Hela](image)

**G**

Relative RNA expression

![Expression levels in Mock and RNase R-treated SiHa](image)
A circBank

hsa-miR-200a-3p
hsa-miR-766-5p
hsa-miR-141-3p
hsa-miR-4677-3p
hsa-miR-122-5p
hsa-miR-500b-5p

starBase

B

Hela

miRNA enrichment

Relative miRNA enrichment

C

SiHa

Relative miRNA enrichment

D

circCDK17-WT 5'-AGAAGGCUAUCCCUCACACUCCG-3'

miR-122-5p 3'-GUUUGUGGUAACAGUGUGAGGAGGU-5'

circCDK17-MUT 5'-AGAAGGCUAUCCCUAGUGUGAGGG-3'

E

Hela

Relative expression of miR-122-5p

F

Hela

Relative luciferase activity

G

SiHa

Relative luciferase activity

H

Relative expression of miR-122-5p

I

Relative miR-122-5p expression

J

Relative expression of miR-122-5p

r = -0.6134

P < 0.001

55

55

CC

Normal