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Original Article

N6-Methyladenosine modified circ-NAB1 modulates cell cycle and epithelial-mesenchymal transition via CDKN3 in endometrial cancer

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Abstract

Endometrial cancer (EC) is a common malignant tumor in the female reproductive system. Circular RNAs (circRNAs) and N6-methyladenosine (m^6A) modification are widely involved in cancer progression. Nevertheless, the cross-talk between circ-NAB1 and m^6A as well as the biological functions of circ-NAB1 in EC remain unclear. Circ-NAB1 was observed to be upregulated in EC tissues and cells by RT-qPCR. MeRIP and RNA pull-down assays were utilized for detecting the m^6A modification of circ-NAB1. The interaction between circ-NAB1 and RNAs was also detected. Colony formation, transwell, flow cytometry, and western blot were utilized for measuring EC cell behaviors. Mechanically, we proved the m^6A demethylase alkylation repair homolog protein 5 (ALKBH5) can mediate circ-NAB1 expression through an m^6A-YTHDF2-dependent manner. Circ-NAB1 overexpression can promote cell proliferation, migration, invasion, epithelial-mesenchymal transition (EMT) process, and cell cycle through functional assays. Circ-NAB1 knockdown exerts the opposite function on EC cells. Furthermore, we proved that circ-NAB1 can sponge miR-876-3p to upregulate the target gene cyclin-dependent kinase inhibitor 3 (CDKN3) in EC cells. CDKN3 overexpression can reverse the impacts of circ-NAB1 depletion on EC cell behaviors. Collectively, we proved that ALKBH5-mediated m^6A modification of circ-NAB1 promoted EMT process and cell cycle in EC via targeting the miR-876-3p/CDKN3 axis.

Keywords: ALKBH5, Circ-NAB1, CDKN3, Endometrial cancer, N6-methyladenosine

1. Introduction

Endometrial cancer (EC) is a common malignant tumor in the female reproductive system with high incidence [1]. Risk factors for EC include obesity, diabetes, estrogen imbalance, untimely menarche, the utilization of tamoxifen, and so on [2]. Recurrence and metastasis are key stages in the formation and development of EC and are also the main causes of death [3]. Currently, common treatment strategies include surgery, chemotherapy, and radiation therapy, which can significantly prolong the survival of early EC patients [4]. However, the efficacy is not significant for recurrent and metastatic EC patients. Thus, it is necessary to investigate EC pathogenesis and develop more effective therapeutic targets for promoting the survival of patients.

Epigenetic modifications have been confirmed to exert vital functions in tumor occurrence and development [5]. N6-methyladenosine (m^6A) is the most prevalent modification of eukaryotic mRNAs, which can also modulate the generation and functions of noncoding RNAs, including circRNAs [6, 7]. The m^6A modification can affect a series of cellular processes, such as pre-mRNA splicing, nuclear transport, mRNA stabilization and translation [8]. The modification is modulated via different regulators, including writers, erasers and readers, which can effectively install and remove mRNA methylation [9]. The writers promote m^6A methylation and comprise of m^6A methyltransferases METTL3, METTL5, METTL14, and other subunits. Erasers are demethylases and include alkylation repair homolog protein 5 (ALKBH5) and FTO. Readers recognize m^6A-methylated transcripts and include YTHDF1, YTHDF2, and YTHDF3. These regulatory proteins are often dysregulated in human cancers and play vital functions in promoting or inhibiting cancer development via modulating downstream targets and signals [10]. Accumulating research efforts have confirmed that m^6A modification can modulate cancer development by circRNAs. For example, m^6A-mediated circ-MDK overexpression facilitates cell proliferation and invasion in hepatocellular carcinoma [11]. ALKBH5-mediated m^6A modification of circ-CCDC134 accelerates metastasis in cervical cancer through the enhancement of HIF1A transcription [12]. Nevertheless, the function of m^6A modification in EC and its latent regulatory mechanism for circRNAs are unclear.

CircRNAs are the class of noncoding RNAs and are mainly derived from exonic regions of protein-coding genes [13]. Different from linear RNAs, circRNAs possess a stable circular structure without 5′ to 3′ polarity [14, 15]. Numerous studies have shown that circRNAs have a broad

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impact on various physiological processes in humans. Furthermore, anomalous expression of circRNAs can affect the progression of human cancers. Circ_104348 is reported to be overexpressed in hepatocellular carcinoma and promote cancer progression through Wnt pathway [16]. Circ-ANAPC7 is lowly expressed in pancreatic cancer and its upregulation can suppress tumor growth through PHLPP2-AKT-TGF-β signaling [17]. Furthermore, some circRNAs have been identified in EC and they take part in modulating EC development. For instance, circ-ESRP1 has been confirmed to enhance metastasis and epithelial-mesenchymal transition (EMT) in EC via the miR-874-3p/CPEB4 axis [18]. Circ_0002577 can accelerate EC cell proliferative capability through IGF1R/PI3K/Akt pathway [19]. Circ- NAB1 (hsa_circ_0002024) is a circRNA formed by exon 4-6 splicing of the host gene NGFI-A binding protein 1 (NAB1). The high-throughput sequencing results [20] identified circ-NAB1 as an abnormally expressed circRNA in EC tissues. However, its specific function in EC remains unclear.

Therefore, this study aimed to investigate the biological function and molecular mechanism of circ-NAB1 in EC, which may provide a novel therapeutic target for EC.

2. Materials and methods

2.1. Tissue collection

EC tissues and normal endometrial tissues were obtained from 15 EC patients who received the operative treatment at the Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi People's Hospital, Wuxi Medical Center, Nanjing Medical University. These tissues were removed and rapidly frozen in liquid nitrogen after resection. All participants provided written informed consent. This project was approved by the Ethics Committee of the Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi People's Hospital, Wuxi Medical Center, Nanjing Medical University.

2.2. Cell culture

EC cell lines (HEC-1-B, Ishikawa, HEC-1-A, and RL95-2), the human endometrial epithelial cell line (hEEC), and 293T cells (ATCC, Manassas, VA, USA) were incubated in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) with 10% FBS at 37°C with 5% CO_2.

2.3. Cell transfection

The specific shRNA targeting circ-NAB1 (sh-circ-NAB1), ALKBH5 (sh-ALKBH5), or YTHDF2 (sh-YTHDF2), as well as their negative control (sh-NC), were purchased from Gene-Pharma (Shanghai, China). For overexpressing circ-NAB1, ALKBH5, YTHDF2, or CDKN3, the full-length sequence was inserted into pcDNA3.1 vector (Geenseed Biotech, Guangzhou, China). The miR-876-3p mimics/inhibitor and NC mimics/inhibitor were synthesized by RiboBio (Guangzhou, China). Lipofectamine 3000 (Invitrogen, USA) was utilized for cell transfection for 48 h.

2.4. RT-qPCR

The total RNA was subjected to extraction by TRIzol (Invitrogen). Then the RNA was reverse transcribed into cDNA utilizing the ReverTra Ace qPCR RT Kit (Toyobo, Japan). The qPCR was implemented via THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan) on the LightCycler 480 Real-Time PCR system (Roche, Shanghai, China). Gene expression was measured by the $2^{-\Delta\Delta Ct}$ method and normalized to U6 or GAPDH.

2.5. Oligo dT (18T) primers and random 6 primers assay

All RNAs were subjected to reverse transcription utilizing random 6 primers. RNAs that possessed poly-A tails were subjected to reverse transcription utilizing Oligo dT primers. For identifying circ-NAB1 tested through RT-qPCR, the Evo M-MLV RT Kit (Accurate Biology, China) was applied in accordance with user guides.

2.6. Western blot analysis

Total protein was subjected to extraction utilizing RIPA (GenePharma). Proteins were isolated by 10% SDS-PAGE and then transferred onto a PVDF membrane. Next, the membranes were blockaded with 5% non-fat milk and then cultured with the primary antibodies (Abcam) at 4°C for one night. Membranes were then cultured with secondary antibody (Abcam). The bands were visualized by an ECL kit (Millipore) and analyzed by ImageJ (v1.8.0; National Institutes of Health).

2.7. Subcellular fraction assay

Nuclear and cytoplasmic RNA was subjected to isolation through the nuclear or cytoplasmic RNA Purification Kit (Thermo Fisher Scientific, USA). Cells were lysed on ice with cell fraction buffer. The fractions were isolated into the supernatant and precipitation after centrifugation. Then the obtained RNA was subjected to extraction and then they were analyzed by RT-qPCR.

2.8. Fluorescent in situ hybridization (FISH)

The circ-NAB1 or miR-187-3p probe was synthesized by GenePharma (Shanghai, China). The hybridization was implemented for a whole night with the probes utilizing the fluorescent in situ hybridization kit in accordance with user guides (GenePharma). The nucleus was dyed by DAPI. The laser-scanning microscope (LSM 780, Zeiss, Germany) was applied to capture images.

2.9. RNA pull-down assay

The biotinylated circ-NAB1 or miR-876-3p probe was synthesized by GenePharma (Shanghai, China) and utilized for culturing with streptavidin agarose beads (Thermo Scientific). Cells were lysed, and the lysate with biotinylated probes was cultured at 4°C overnight. RNA complex combined with the beads was subjected to elution, and western blot or RT-qPCR was utilized to estimate gene enrichment.

2.10. RNA immunoprecipitation (RIP) assay

In accordance with user guides, the Magna RIP kit (Millipore, Billerica, MA, USA) was applied for this assay. The cell lysate was treated with RIP buffer comprising magnetic beads conjugated with antibodies against ALKBH5, YTHDF2, or AGO2 (Millipore), as well as the control IgG. Beads were rinsed, and the complex was cultured with proteinase K to remove proteins. The enrichment was analyzed by RT-qPCR. Methylated RIP was implemented through a MeRIP kit (BersinBio, Guangzhou, China) in accordance with user guides, and the anti-m^6A (Abcam, Cambridge, USA) was applied for MeRIP.

2.11. Luciferase reporter assay

The wild-type (WT) and mutant (Mut) m^{6}A or 3'UTR of circ-NAB1 were designed and the fragment sequences were synthesized. Then the fragments comprising binding sites were inserted into the luciferase vector (Ribobio). Next, 293T cells were incubated in 24-well plates and transfected with 200 ng of luciferase vectors with indicated plasmids by Lipofectamine 3000 (Invitrogen). After 48 h, Dual Luciferase Reporter Assay system (Promega) was utilized to determine the luciferase activities.

2.12. RNA stability assay

After transfection, cells were treated with ActD (5 μg/ml) for 24 h, 48 h, or 72 h. Then, cells were gathered and the total RNA was obtained by the Trizol reagent, followed by the analysis of RT-qPCR.

2.13. Colony formation

Cells (500 cells/well) were put into 6-well plates 48 h and cultured for two weeks. Then, they were fixed with 4% formaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich). Colonies were photographed and accumulated.

2.14. Transwell assay

The transfected cells in 200 μL of RPMI-1640 medium were supplemented to the upper 24-well Transwell chambers (Corning, NY, USA). The Transwell chambers precoated with Matrigel were employed for invasion assay. The bottom chambers were full of 600 μL RPMI-1640 supplemented with 10% FBS. After 24 h, cells were subjected to fixation with 4% paraformaldehyde and dyed with 0.5% crystal violet solution (Sigma-Aldrich). The inverted microscope (Olympus) was utilized for observation.

2.15. Flow cytometry analysis

The transfected cells were rinsed by PBS and fixed in ice-cold 70% ethanol for one night, followed by dyeing with RNase (50 μg/ml) and 50 μg/ml propidium iodide (BD Biosciences, San Jose, CA, USA) for half an hour at 4 °C. After that, cell cycle was estimated by flow cytometry (FACSCalibur, BD Biosciences) and FlowJo software (BD Biosciences).

2.16. Statistical analysis

GraphPad Prism software (version 7.0, USA) was applied to analyze the data, which were represented as means ± SD from three individual repeats. Group comparison was implemented by Student's t-test or one-way ANOVA. P<0.05 represented statistical significance.

3. Results

3.1. ALKBH5 mediates RNA methylation modification of circ-NAB1

The high-throughput sequencing results [20] showed the circRNAs with abnormal expression in EC, and the top 5 circRNAs (circ-SEC63, circ-ARHGEF12, circ-ASXL1, circ-NAB1, and circ-DEK) with the most significant upregulation were selected for our study. As shown by RT-qPCR results, 4 circRNAs expressed at a high level in tumor tissues, but circ-NAB1 upregulation was the most significant (Fig. 1A). According to the analysis of circPrimer prediction website (https://www.bio-inf.cn/), we found that circ-NAB1 is formed by exon 4-6 splicing of NAB1 (Fig. 1B). Next, RT-qPCR was applied for testing circ-NAB1 expression in different EC cells and control cells hEEC, and the upregulation of circ-NAB1 in EC cells was further verified (Fig. 1C). Given the highest expression of circ-NAB1 in RL95-2 and Ishikawa cells, they were chosen for later assays. We validated the circular feature of circ-NAB1 in cells. After the amplification of random primer or Oligo dT primer, we observed from RT-qPCR results that circ-NAB1 expression was markedly reduced in the Oligo dT primer group in cells (Fig. 1D). Thus, we verified the circular properties of circ-NAB1 in EC cells. Additionally, the FISH assay and subcellular fraction assay illustrated that circ-NAB1 was primarily distributed in the cytoplasm (Fig. 1E-F). Overall, circ-NAB1 was expressed at a high level in EC cells and tissues.

The regulatory mechanism of circ-NAB1 overexpression in EC was further investigated. It is reported that m^{6}A modification exerts a vital function in post-transcriptional regulation of circRNAs [21]. Through SRAMP (http://www.cuilab.cn/sramp/), we found that circ-NAB1 had three m^{6}A modification sites with high confidence at 240, 385, and 660 (Fig. 1G). Then we treated cells with the methylation inhibitor DAA and control DMSO, and RT-qPCR illustrated circ-NAB1 expression was elevated

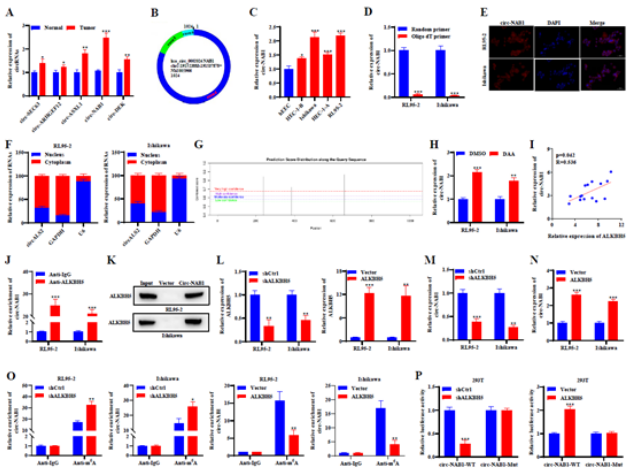

Fig. 1. ALKBH5 mediates RNA methylation modification of circ-NAB1. (A) Circ-SEC63, circ-ARHGEF12, circ-ASXL1, circ-NAB1, and circ-DEK expression in normal and tumor tissues was tested through RT-qPCR. (B) Scheme illustrates the formation of circ-NAB1. (C) Circ-NAB1 expression in EC cells and normal cells was tested through RT-qPCR. (D) Circ-NAB1 expression in RL95-2 and Ishikawa cells was tested through RT-qPCR after the amplification of random primer or Oligo dT primer. (E-F) FISH and the subcellular fraction assays were implemented to estimate circ-NAB1 subcellular location. (G) Three m^{6}A modification sites for circ-NAB1 were predicted by SRAMP website. (H) Circ-NAB1 expression in DMSO or DAA-treated cells was determined through RT-qPCR. (I) The correlation between circ-NAB1 and ALKBH5 expression in EC tissues (n=15). (J-K) RIP and RNA pull-down assays were applied for detecting the combination of circ-NAB1 and ALKBH5. (L) RT-qPCR results of the knockdown efficiency and overexpression efficiency of ALKBH5 in cells. (M-N) RT-qPCR outcomes of circ-NAB1 expression after ALKBH5 was overexpressed or decreased in cells. (O) MeRIP assay was utilized to assess the m^{6}A status of circ-NAB1 when ALKBH5 was silenced or overexpressed. (P) Luciferase reporter assay was utilized for assessing the interplay of circ-NAB1 and ALKBH5. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

by DAA stimulation (Fig. 1H). ALKBH5 is a representative m^{6}A demethylase [22], and we detected the correlation between ALKBH5 and circ-NAB1 in EC tissues. As a result, circ-NAB1 was positively correlated with ALKBH5 in tissues (Fig. 1I). Then, we further estimated the interaction of circ-NAB1 and ALKBH5 by RIP assay. The results manifested that ALKBH5 was abundantly enriched by anti-ALKBH5 (Fig. 1J). RNA pull-down assay further verified that ALKBH5 can be pulled by bio-circ-NAB1, suggesting circ-NAB1 interacted with ALKBH5 (Fig. 1K). Then we constructed the ALKBH5 knockdown or overexpression cell lines by transfecting the sh-ALKBH5 plasmids or pcDNA3.1-ALKBH5 vectors, and RT-qPCR confirmed the transfection efficiency (Fig. 1L). Next, we observed that circ-NAB1 expression was declined via ALKBH5 knockdown and elevated via ALKBH5 upregulation (Fig. 1M-N). Subsequently, Me-RIP was implemented to further determine the m^{6}A status of circ-NAB1. We discovered that circ-NAB1 enrichment in the anti-m^{6}A group was notably increased by ALKBH5 depletion while decreasing by ALKBH5 overexpression (Fig. 1O). Further, we observed the luciferase activity of circ-NAB1-WT was declined via ALKBH5 silencing and elevated by ALKBH5 overexpression. However, there was no notable alteration in luciferase activity after mutation at the m^{6}A site of circ-NAB1 in control cells and ALKBH5-overexpressed cells (Fig. 1P). These results confirmed that ALKBH5 mediated the m^{6}A methylation to increase circ-NAB1 expression in EC.

3.2. ALKBH5 stabilizes circ-NAB1 through YTHDF2

YTHDF2 is the reader protein in m^{6}A methylation that can bind to m^{6}A-modified RNAs, mainly increasing the decay of m^{6}A methylated transcript [23, 24]. RIP and RNA pull-down assays manifested YTHDF2 can combine with circ-NAB1 in cells (Fig. 2A-B). We silenced or increased YTHDF2 expression in cells, and the knockdown efficiency and overexpression efficiency of YTHDF2 were verified by RT-qPCR (Fig. 2C). Circ-NAB1 expression was observed to be declined by YTHDF2 overexpression, while increasing by YTHDF2 depletion, suggesting the negative correlation between them (Fig. 2D). Additionally, circ-NAB1-WT luciferase activity was found to be promoted via YTHDF2 silencing and decreased by YTHDF2 upregulation, which further verified the combination of circ-NAB1 and YTHDF2 (Fig. 2E). Next, cells were treated with Act D for performing the stability assay. Through RT-qPCR, we discovered that the degree of degradation of circ-NAB1 was significantly enhanced in cells overexpressed with YTHDF2 (Fig. 2F). Furthermore, we detected the impact of ALKBH5 and YTHDF2 downregulation on circ-NAB1 expression. Circ-NAB1 expression was notably declined via ALKBH5 depletion while recovering by YTHDF2 silencing (Fig. 2G). Overall, these results proved that ALKBH5 increased circ-NAB1 expression by enhancing its stability via YTHDF2.

3.3. Circ-NAB1 regulates cell proliferation, EMT process, and cell cycle in EC

The biological functions of circ-NAB1 in EC cells were further studied. We constructed the circ-NAB1 knockdown cell line or circ-NAB1 overexpression cell line by the transfection of sh-circ-NAB1 or pcDNA3.1-circ-NAB1. RT-qPCR verified the transfection efficiency

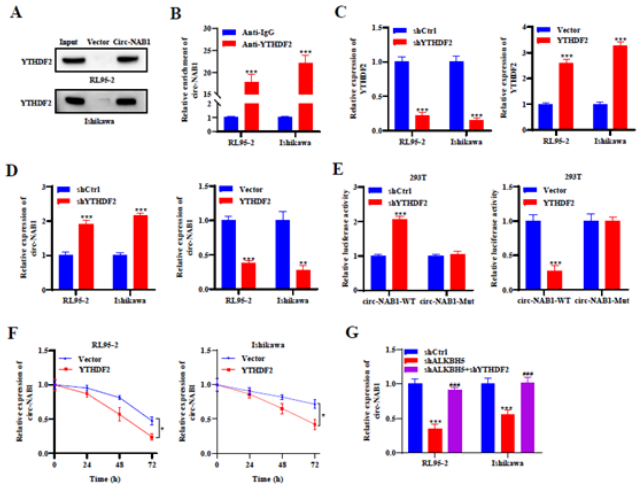

Fig. 2. ALKBH5 stabilizes circ-NAB1 through YTHDF2. (A-B) RNA pull-down and RIP assays were employed for estimating the interaction of circ-NAB1 and YTHDF2. (C) RT-qPCR results of the knockdown efficiency and overexpression efficiency of YTHDF2 in cells. (D) Circ-NAB1 expression was tested through RT-qPCR when YTHDF2 was suppressed or upregulated. (E) Luciferase reporter assay was utilized for assessing the interplay of circ-NAB1 and YTHDF2. (F) Circ-NAB1 expression and stability were tested by RT-qPCR in Act D-treated cells when YTHDF2 was silenced or upregulated. (G) Circ-NAB1 expression was tested through RT-qPCR in cells when ALKBH5 and YTHDF2 were silenced. $^{***}p < 0.001$; $^{###}p < 0.001$.

(Fig. 3A). Then colony formation assay manifested that circ-NAB1 depletion markedly reduced the quantity of colonies while circ-NAB1 overexpression promoted it, suggesting cell proliferative capability was promoted by circ-NAB1 overexpression (Fig. 3B). Next, it was illustrated by transwell assays that cell migratory and invasive capabilities were reduced by circ-NAB1 depletion but enhanced by circ-NAB1 overexpression (Fig. 3C-E). Then the levels of proteins related to EMT process were assessed through western blot. E-cadherin levels were elevated by circ-NAB1 silencing, whereas N-cadherin and Vimentin levels were reduced by that. Nevertheless, circ-NAB1 overexpression showed the opposite function on E-cadherin, N-cadherin, and Vimentin levels (Fig. 3F-G). These results proved that EMT process could be facilitated by circ-NAB1 upregulation and suppressed by circ-NAB1 inhibition. Additionally, the impact of circ-NAB1 on cell cycle was estimated via flow cytometry. We observed that circ-NAB1 depletion caused the G0/G1 arrest in cells, while circ-NAB1 overexpression exerted the opposite function (Fig. 3H-I). Thus, we confirmed that circ-NAB1 functions as the oncogene in EC.

3.4. Circ-NAB1 acts as a molecular sponge for miR-876-3p, and CDKN3 is targeted by miR-876-3p

The regulatory mechanism mediated by circ-NAB1 in EC cells was investigated. Through RIP assay, we observed that, in comparison of control IgG, circ-NAB1 was preferentially enriched in RISC complex containing Ago2, suggesting circ-NAB1 may function as a sponge for microRNAs (miRNAs) (Fig. 4A). Through the prediction of circBank (http://www.circbank.cn/) and DEmiRNA, we obtained three possible miRNAs (miR-136-3p, miR-876-3p, and miR-1290) that may bind to circ-NAB1 (Fig. 4B). DEmiRNA refers to the miRNAs that are abnormally ex-

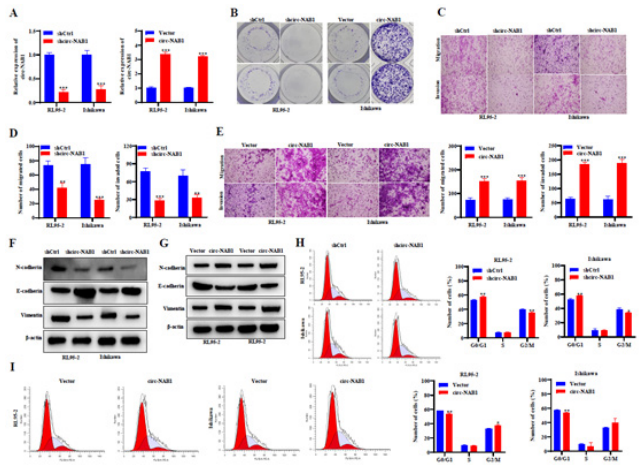

Fig. 3. Circ-NAB1 regulates EC cell proliferation, EMT process, and cell cycle. (A) RT-qPCR results of the knockdown efficiency and overexpression efficiency of circ-NAB1 in cells. (B) Cell proliferative capability was determined through colony formation assay when circ-NAB1 was silenced or overexpressed. (C-E) Cell proliferative and migratory capabilities were evaluated through transwell assays when circ-NAB1 was suppressed or overexpressed. (F-G) E-cadherin, N-cadherin, and Vimentin levels were measured through western blot. (H-I) Flow cytometry was applied to determine cell cycle in different groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

pressed in EC in accordance with the GSE35794 dataset. For screening, we observed that miR-876-3p possessed the highest enrichment in the Bio-circ-NAB1 group (Fig. 4C). Thus, we selected miR-876-3p for further assays. FISH assay showed that circ-NAB1 and miR-876-3p were mainly distributed in cytoplasm (Fig. 4D). The luciferase activity of circ-NAB1 was declined via miR-876-3p upregulation but elevated via miR-876-3p depletion, which indicated that circ-NAB1 could combine with miR-876-3p (Fig. 4E). Overall, circ-NAB1 can sponge miR-876-3p in EC cells.

The miRDB database (https://mirdb.org/) was further utilized to predict the candidate mRNAs that may bind to miR-876-3p. The GSE17025 dataset was utilized for analyzing the differentially expressed genes in EC samples. Through the analysis of both miRDB and GSE17025, we obtained 99 possible mRNAs for miR-876-3p (Fig. 4F). Then GO analysis showed the five most significant enrichment processes of genes in MF, CC, and BP, among which we found that genes were significantly enriched in biological processes such as cell cycle (Fig. 4G). Then we established the protein-protein interaction networks (PPI) to evaluate the biological interaction of genes through the String database, which included 53 nodes and 52 edges (Fig. 4H). Then, we utilized the cytoHubba plugin of Cytoscape to determine the hub genes. Based on the MCC (Maximal Clique Centrality), the top five hub genes (CENPN, MKI67, EGFR, KIF20, and CDKN3) were selected. The highest scoring protein is CDKN3, which is highly correlated with tumor cell cycle (Fig. 4I). Thus, CDKN3 was considered as the target gene. GEPIA database showed that CDKN3 was expressed at a high level in Uterine Corpus Endometrial Carcinoma (UCEC) tissues (Fig. 4J). The survival curve analyzed by KMplot website (https://kmplot.com/analysis/) illustrated that CDKN3 high expression represented the poor prognosis of UCEC patients (Fig. 4K). Then, we overexpressed or silenced miR-876-3p expression in EC cells. We further observed CDKN3 expression levels declined via miR-876-3p upregulation while increasing via miR-876-3p inhibition (Fig. 4L-M). RNA pull-down assay verified CDKN3 can be abundantly pulled down by the biotinylated miR-876-3p probe, suggesting the combination between them (Fig. 4N). Moreover, we observed that CDKN3 expression can be declined by circ-NAB1 depletion while reversing by miR-876-3p inhibition (Fig. 4O). Thus, CDKN3 was the target gene for miR-876-3p in EC.

3.5. Circ-NAB1 facilitates EC progression by upregulating CDKN3

The rescue assays were finally performed to determine the impact of circ-NAB1 and CDKN3 on EC progression. CDKN3 expression was upregulated in RL95-2 cells (Fig. 5A). We discovered that cell proliferative capability was reduced by circ-NAB1 silencing, while CDKN3 upregulation counteracted this effect (Fig. 5B). Western blot results illustrated that the E-cadherin levels promoted by circ-NAB1 knockdown were reduced by CDKN3 overexpression, and N-cadherin and Vimentin levels inhibited by circ-NAB1 knockdown were increased by CDKN3 ove-

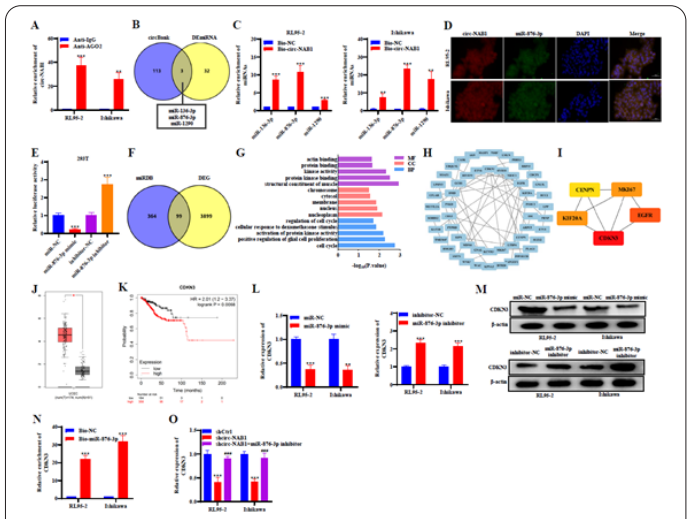

Fig. 4. Circ-NAB1 can sponge miR-876-3p in EC, and CDKN3 is targeted by miR-876-3p. (A) RIP assay was employed for testing the combination of circ-NAB1 and Ago2 protein in cells. (B) Venn diagram showed the intersection of circBank and DEmiRNA for the possible miRNAs of circ-NAB1. (C) RNA pull-down assay was performed to estimate the combination of circ-NAB1 and miR-136-3p/miR-876-3p/miR-1290. (D) The co-location of circ-NAB1 and miR-876-3p in cells was tested through FISH assay. (E) Luciferase reporter assay was implemented to determine the combination of circ-NAB1 and miR-876-3p. (F) The miRDB and GSE17025 databases were utilized to screen out the candidate mRNAs that may bind to miR-876-3p and are highly expressed in EC samples. (G) GO analysis was carried out to measure the biological functions of these candidate mRNAs. (H) PPI network was established by the String database. (I) The hub genes were screened by the cytoHubba plugin of Cytoscape. (J) CDKN3 expression in UCEC tissues was predicted by GEPIA database. (K) The survival rate of UCEC patients with high/low CDKN3 was predicted by KMplot database. (L-M) RT-qPCR and western blot outcomes of CDKN3 expression in cells transfected with miR-876-3p mimics or inhibitors. (N) RNA pull-down assay was utilized to determine the combination of miR-876-3p and CDKN3. (O) RT-qPCR outcomes of CDKN3 expression when miR-876-3p and circ-NAB1 were silenced. $^{**}p < 0.01$, $^{***}p < 0.001$; $^{\#\#\#}p < 0.001$.

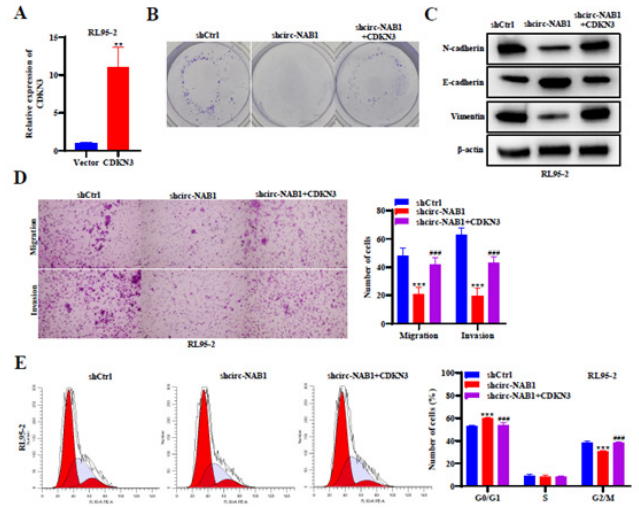

Fig. 5. Circ-NAB1 facilitates EC progression by upregulating CDKN3. (A) RT-qPCR outcomes of overexpression efficiency of CDKN3. (B) Cell proliferative capability was tested through colony formation assay in the sh-control group, the sh-circ-NAB1 group, and the sh-circ-NAB1+CDKN3 group. (C) Western blot outcomes of E-cadherin, N-cadherin and Vimentin levels in cells. (D) Cell migratory and invasive capabilities were estimated through transwell assays. (E) Flow cytometry analysis was utilized to determine cell cycle. $^{**}p < 0.01$, $^{***}p < 0.001$; $^{\#\#\#}p < 0.001$.

rexpression (Fig. 5C). Furthermore, we discovered the inhibitory function of circ-NAB1 knockdown on cell migration and invasion was abolished via CDKN3 upregulation (Fig. 5D). Then, it was manifested by flow cytometry analysis that cell cycle suppressed by circ-NAB1 silencing was recovered by CDKN3 upregulation (Fig. 5E). Thus, we confirmed that circ-NAB1 facilitated EC progression by upregulating CDKN3.

4. Discussion

EC has become a huge problem for women's health all over the world [1]. Current conventional therapies have minimal effect on patients with advanced EC and fail to reduce the high mortality rate of patients [1]. In recent years, research efforts on targeted therapies have provided a promising clinical strategy for EC treatment [25]. There is growing evidence that circRNAs are involved in human tumor progression and play an important regulatory function [26]. In EC, some dysregulated circRNAs were also identified, including circ-ATAD1 [27], circ-ZNF608 [28], circ-PUM1 [29], and so on. The high-throughput sequencing results have [20] identified circ-NAB1 as an abnormally expressed circRNA in EC tissues. Similarly, our study proved circ-NAB1 expressed at a high level in EC tissues and cells. Knocking down circ-NAB1 notably suppressed cell proliferative, migratory, invasive capabilities, EMT processes, and cell cycle. Circ-NAB1 overexpression had the opposite effect on EC cell behaviors. Thus, we proved that circ-NAB1 can function as an oncogene in EC.

Studies have confirmed that m^{6}A is a novel epigenetic regulation that can modulate cell growth and differentiation via regulating RNA splicing, translation, and stability [30]. The methyltransferases are responsible for m^{6}A methylation, which is recognized by the reader proteins and erased by demethylases [31]. ALKBH5 is a common demethylase [32]. A flow of studies has confirmed that ALKBH5-mediated m^{6}A modification exerts a crucial function in cancer development. ALKBH5-mediated m^{6}A modification of circ-CCDC134 promotes cervical cancer metastasis via promoting HIF1A expression [12]. ALKBH5 inhibits cell invasion in gastric cancer through PKMYT1 m^{6}A modification [33]. ALKBH5 facilitates cell proliferative capability in head and neck squamous cell carcinoma via suppressing RIG-I [34]. Furthermore, ALKBH5-mediated m^{6}A modification of SOX2 maintains the tumorigenicity potential of endometrial cancer stem cells [35]. Bioinformatics tools showed that circ-NAB1 had m^{6}A modification sites. Our study further proved that circ-NAB1 was positively correlated with ALKBH5 in EC tissues and combined with ALKBH5. MeRIP verified that ALKBH5 upregulation could reduce the m^{6}A level of circ-NAB1. Overall, we confirmed that ALKBH5-mediated the m^{6}A methylation of circ-NAB1 resulted in the overexpression of circ-NAB1 in EC. M^{6}A modification is mainly recognized via reader proteins (YTHDF1/2/3), which can affect RNA translation, stability or splicing [36, 37]. YTHDF1 and YTHDF3 exert the roles of translation modulation, whereas YTHDF2 can promote the decay of m^{6}A methylated transcript [24, 38, 39]. More and more studies have revealed that YTHDF2 can play a regulatory function in breast cancer [40], liver cancer [41], EC [42] and so on. Herein, we found that YTHDF2 was negatively correlated with circ-NAB1 and can combine with circ-NAB1. Circ-NAB1 degradation in EC cells was enhanced by YTHDF2 upregulation. Furthermore, circ-NAB1 expression reduced by ALKBH5 depletion was recovered by YTHDF2 silencing. Thus, we confirmed that ALKBH5 increased circ-NAB1 expression by the m^{6}A-YTHDF2-dependent mechanism. A similar regulatory mechanism has also been confirmed in other tumors. Guo et al. suggest ALKBH5 inhibits pancreatic cancer cell growth through activating PER1 in an m^{6}A-YTHDF2-dependent manner [43]. Yang et al. reveal that ALKBH5 modulates STAT3 activation to influence the progression of osteosarcoma by the m^{6}A-YTHDF2-dependent manner [44]. These studies further supported our findings.

Circ-RNA can function as a ceRNA to sponge miRNAs and secure their inhibitory effect on the expression of targets, thereby forming a circ-RNA-miRNA-mRNA regulatory network [45]. This regulatory network has been verified in assorted human cancers, including EC [46]. CircRNA_0088036 can function as a ceRNA to facilitate bladder cancer cell proliferation by sponging miR-140-3p [47]. Additionally, circ-ESRP1 accelerates metastasis and EMT process in EC via the miR-874-3p/CPEB4 axis [18]. MiR-876-3p has been confirmed to be a tumor suppressor by some existing research. MiR-876-3p can inhibit colon cancer development [48]. MiR-876-3p targets KIF20A to repress cell proliferation and tumor growth of glioma [49]. MiR-876-3p can be sponged by circ-RNF111 and its overexpression represses the malignancy of gastric cancer [50]. Herein, the bioinformatics and experimental results proved that circ-NAB1 can sponge miR-876-3p in EC.

CDKN3 is a member of the protein phosphatases family and exerts a crucial function in modulating cell cycle and proliferation [51]. CDKN3 plays its role via combining with cyclin proteins, which leads to CDK1 and CDK2 dephosphorylation and cell cycle suppression [52]. CDKN3 overexpression and its oncogenic function

have been extensively explored in different cancers. For example, CDKN3 upregulation facilitates cell growth and invasion of ovarian cancer [53]. CDKN3 promotes cell proliferative capability and cell cycle in prostate cancer [54]. CDKN3 expedites the development of esophageal squamous cell carcinoma [55]. Herein, we proved CDKN3 could bind to miR-876-3p in EC cells and be positively regulated by circ-NAB1. CDKN3 overexpression notably reversed the function of circ-NAB1 depletion on EC cells and facilitated cell malignant phenotypes.

5. Conclusion

Taken together, this study confirmed that ALKBH5-mediated m^{6}A modification of circ-NAB1 promotes cell proliferative, migratory, invasive capabilities, EMT, and cell cycle in EC via targeting the miR-876-3p/CDKN3 axis. These discoveries may offer novel therapeutic targets for EC.

Conflict of Interests

The authors declare no competing interests.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

We have received approval from the Ethics Committee of the Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi People's Hospital, Wuxi Medical Center, Nanjing Medical University.

Informed Consent

We have received informed consent from the Ethics Committee of the Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi People's Hospital, Wuxi Medical Center, Nanjing Medical University.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

ZY and ZJ contributed to the study conception and design. Experimental operation, data collection and analysis were performed by ZJ. The first draft of the manuscript was written by ZJ. All authors commented on previous versions of the manuscript.

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