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Original Article

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CircSMAD2 accelerates endometrial cancer cell proliferation and metastasis by regulating the miR-1277-5p/MFGE8 axis

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ABSTRACT

Objective: Endometrial cancer (EC) is a common gynecological malignant tumor. CircRNAs play crucial roles in cancer progression and metastasis. However, the biological functions of circRNAs in EC remain largely unknown.
Methods: CircSMAD2, miR-1277-5p, MFGE8 and relative maker protein expression in EC tissues or cell lines were analyzed by quantitative real-time polymerase chain reaction and Western blot. In vitro and in vivo functional assays, including EDU, CCK8, colony formation, transwell, tube formation and tumor xenograft assays, were conduct to explore the effects of circSMAD2 on EC. Mechanism assays were conducted to confirm the binding between miR-1277-5p and circSMAD2 or MFGE8 expression.
Results: Upregulation of circSMAD2 was uncovered in both EC tissues and cell lines. Functionally, silencing of circSMAD2 apparently inhibited the proliferation, migration, invasion and angiogenesis of EC cell lines in vitro. Mechanistically, circSMAD2 sponged miR-1277-5p to upregulate MFGE8 expression. The decrease of miR-1277-5p and increase of MFGE8 were observed both in EC tissues and cell lines. Then MFGE8 knockdown or miR-1277-5p upregulation suppressed EC cell oncogenic biological behavior. Rescue experiments showed that miR-1277-5p mimics countervailed the anticancer effects of circSMAD2 silencing on EC. Besides that, MFGE8 overexpression also attenuated the inhibitory action of miR-1277-5p mimic in EC. Moreover, knockdown of circSMAD2 inhibited EC growth in vivo.
Conclusion: CircSMAD2 functions as an oncogene in promoting the progression of EC through miR-1277-5p/MFGE8 axis.

Keywords: Circular RNA SMAD Family Member 2; MicroRNA-1277-5p; Milk Fat Globule Epidermal Growth Factor 8; Endometrial Cancer

Synopsis

We analyzed the potential role of circSMAD2 in endometrial cancer (EC) and revealed that circSMAD2 promoted EC malignant progression by sponging miR-1277-5p and consequently promoting MFGE8. Our results provided promising therapeutic targets for EC.

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Author Contributions
Conceptualization: W.Y.; Data curation: W.F., S.J., G.X.; Formal analysis: W.F., S.J., G.X.; Funding acquisition: G.X., L.F.; Writing - original draft: W.Y.; Writing - review & editing: L.F.

INTRODUCTION

Endometrial carcinoma (EC) is one of the three major gynecological malignancies [1,2]. There is a trend of younger generation in China [3,4]. Despite many studies on EC over the years, the exact pathogenesis of EC is still not clear. Thus, exploring the possible pathogenesis mechanisms is necessary to identify new biomarkers for diagnosis, treatment and prognosis of EC.

CircRNAs are a new member of the non-coding RNAs. They are stable, conservative, universal and specific [5,6]. Accumulation of evidence reveals that circRNA expression is significantly different during disease development [7-11]. CircRNA can act as a tumor initiating factor or tumor suppressor [12]. Mounting evidence shows circRNA regulates tumor progression by binding to microRNAs (miRNA/miR) [13]. For example, circ_0006988 facilitates NSCLC cell growth by miR-491-5p/MAP3K3 axis [14]. Circular RNA circRHOBTB3 function as a tumor inhibitor in hepatocellular carcinoma through targeting miR-18a [15]. CircRASSF2 may be a prognostic factor of breast cancer, which facilitates breast cancer metastasis through regulating the miR-1205/HOXA1 pathway [16]. In a recent report, circSMAD2 was proved to be upregulated and facilitate the progression of colorectal cancer cells [17]. Additionally, a study revealed that circSMAD2 was an upregulated circRNA in EC samples via circRNA microarray [18]. However, the function and underlying mechanisms of circSMAD2 are still unknown.

MiRNAs also participate in cancer process [19]. As reported, miR-203-3p curbed the metastasis of pancreatic cancer cells by targeting FGF2 [20]. Restoration of miR-148a-3p suppressed cancer cell proliferation through DNMT1 and UTF1 [21]. In our research, we predict that miR-1277-5p has the binding sites with circSMAD2. Thus, we start to investigate the function and mechanisms of miR-1277-5p and circSMAD2 in EC.

Herein, we explored the action of circSMAD2 and miR-1277-5p on EC progression. Besides that, the relationship between circSMAD2 and miR-1277-5p, as well as the potential mechanism underlying them in EC progression were also investigated.

MATERIALS AND METHODS

1. Tissue samples collection

58 EC tissues and matched adjacent normal tissues were recruited from Shanxi Province Cancer Hospital. Tissue specimens were kept at −80°C. The Ethics Committee of Shanxi Province Cancer Hospital approved this study. Each patient provided written informed consent.

2. Cell culture and transfection

Human normal endometrial epithelial cells and five EC cells (AN3CA, Ishikawa, KLE, HEC1-B and HEC1-A) were bought from ATCC (Manassas, VA, USA). All cells were incubated in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) at 37°C.

3. Cell transfection

Ishikawa and HEC1-A cells were selected for cell transfection. Short hairpin RNAs for circSMAD2 (sh-circSMAD2#1, sh-circSMAD2#2, sh-circSMAD2#3) or the control sh-NC were constructed by Invitrogen (Carlsbad, CA, USA). MiR-1277-5p mimics, miR-1277-5p inhibitors, siRNA of MFGE8, pcDNA-MFGE8 and their corresponding negative controls

were obtained from Sangon (Shanghai, China). Then cell transfection was carried our using Lipofectamine 2000 (Invitrogen).

4. Quantitative real-time polymerase chain reaction (qRT-PCR)

cDNA was generated using SuperScript cDNA Kit (Invitrogen). qRT-PCR was performed in PCR System with SYBR Green (Roche Diagnostics, Burgess Hill, UK). GADPH or U6 was used as the internal reference. circSMAD2, miR-1277-5p, and MFGE8 relative expression was measured using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in **Table S1**.

5. Cell counting kit 8 (CCK8) assay

Transfected cells with complete medium were inoculated in a 96-well plate. 10 μL CCK-8 (Bosterbio, Wuhan, China) per well was added, and then incubated at for 2 hours. The absorbance value was recorded by a plate reader at 450 nm.

6. 5-Ethynyl-2'-deoxyuridine (EdU) assay

Transfected cells were inoculated to a 24-well plate, then cell proliferation was performed as per the instruction of EdU cell proliferation kit (Ribobio, Guangzhou, China). Finally, EdU-positive cells were counted in five random fields.

7. Colony formation

Transfected cells were incubated into 6-well plates (500 cells per well) and incubated for 14 days. After fixed using methanol (Bosterbio) for 25 minutes, cells were stained using 0.1% crystal violet (Beyotime, Shanghai, China) for 15 minutes. The colonies were counted using a light microscope (Bio-Rad).

8. Transwell assay

Ishikawa and HEC1-A cells suspended with serum-free medium were seeded into the upper chambers. After incubating for 24 hours, the cells that migrated and invaded from the upper to the bottom were fixed with 4% paraformaldehyde (Beyotime) for 30 minutes. Finally, the pictures were obtained under microscope.

9. HUVECs tubule formation

HUVECs were re-suspended in 100 μL EC cell-conditioned medium and reseeded (3×10^4 cells/well) onto Matrigel-coated wells. After 12 hours, we used a microscope to obtain images.

10. Western blot

After being quantified protein concentration, protein was separated using SDS-PAGE electrophoresis. The membranes were incubated with the primary antibodies, including anti-PCNA (1:1,000, 13110T; Cell Signaling Technology, Danvers, MA, USA), anti-MMP9 (1:500, ab76003; Abcam, Burlingame, CA, USA), anti-VEGFA (1:1,000, ab214424; Abcam), anti-MFGE8 (1:500, AF2767; NOVUS, Littleton, CO, USA), and anti-β-actin (1:4,000, ab8227; Abcam). Next, the corresponding HRP-conjugated secondary antibodies (Abcam) were employed to block the membrane. After 2 hours, the protein band was visualized using ECL chromogenic substrate (Beyotime, Shanghai, China).

11. RNA pull-down assay

Ishikawa and HEC1-A cells were transfected with Bio-miR-1277-5p (RiboBio) or Bio-NC (RiboBio), and then the cell lysates were hatched with streptavidin-coated magnetic beads. Finally, the abundance of miR-1277-5p and circSMAD2 was detected by qRT-PCR.

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12. Dual-luciferase reporter assay

The sequences of circSMAD2 and MFGE8 containing the wild type (WT) and mutant (MUT) miR-1277-5p binding sites were cloned into the pmirGLO vectors to produce the corresponding vectors (circSMAD2-WT/MUT and MFGE8 3'UTR-WT/MUT). Then, Ishikawa and HEC1-A cells were transfected with the reporter vector and miR-1277 mimic or miR-NC. Luciferase activities were tested using Dual-Lucy Assay Kit (Solarbio, Beijing, China).

13. RIP assay

The lysates of Ishikawa and HEC1-A cells were reacted with the magnetic bead (Millipore, Bedford, MA, USA) and anti-AGO2 (1:500, ab186733, Abcam) or control anti-IgG. Then immunoprecipitated RNA was purified, and the enrichment of circSMAD2 and miR-1277-5p were evaluated by qRT-PCR.

14. Mice xenograft models

5 weeks old BALB/c nude mice (Vital River, Beijing, China) were randomly assigned into 2 groups. Ishikawa cells transfected with sh-circSMAD2#1 or sh-NC were resuspended with PBS (5×10^6 cells/200 μL PBS), and then the cell suspensions were subcutaneously injected into mice. Tumor volume was calculated, which was measured from 7 days after injection. The mice were sacrificed and tumor weight was detected after 35 days. MFGE8 and Ki-67 IHC staining using SP Kit (Invitrogen) with anti-MFGE8 (1:200, NBP1-90023, NOVUS) and anti-Ki-67 (1:500, ab92742, Abcam). Our research was permitted by Shanxi Province Cancer Hospital.

15. Statistical analysis

All experiments were repeated three times. GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses. Data were presented as mean ± standard deviation (SD). Student's t-test and one-way analysis of variance were used to analyze differences among groups.

RESULTS

1. Silencing circSMAD2 inhibited EC cell malignancy in vitro

CircSMAD2 expression was overexpressed in both EC tissues and EC cell lines (**Fig. S1A and B**). The high expression of circSMAD2 was correlated with poor overall survival (**Fig. S1C**). Next, circSMAD2 stability was confirmed by RNase R assay (**Fig. S1D**). The three shRNAs of circSMAD2 (especially sh-circSMAD2#1) could significantly reduce circSMAD2 expression, while the level of linear SMAD2 was no changed (**Fig. 1A and B**). Therefore, sh-circSMAD2#1 was used in further analyse. Subsequently, silencing circSMAD2 in Ishikawa and HEC1-A cells greatly inhibited cell proliferation and viability (**Fig. 1C-E**). Then, Ishikawa and HEC1-A cell migration and invasion was reduced after circSMAD2 knockdown (**Fig. 1F and G**). Moreover, downregulated circSMAD2 apparently curbed the angiogenesis of Ishikawa and HEC1-A cells (**Fig. 1H**). Western blot data manifested that knocking down circSMAD2 remarkably decreased PCNA (proliferation marker), MMP2 (metastasis marker) and VEGFA (angiogenesis maker) protein expression (**Fig. 1I**).

2. CircSMAD2 sponged miR-1277-5p

Subsequently, the underlying mechanisms of circSMAD2 in the regulation of EC tumorigenesis was then elucidated. CircSMAD2 predominantly localized to the cytoplasm (**Fig. 2A**). Thus, potential miRNAs interacted with circSMAD2 were detected. We discovered that circSMAD2 had a putative binding site for miR-1277-5p by bioinformatics analysis (**Fig. 2B**). Then, qRT-PCR

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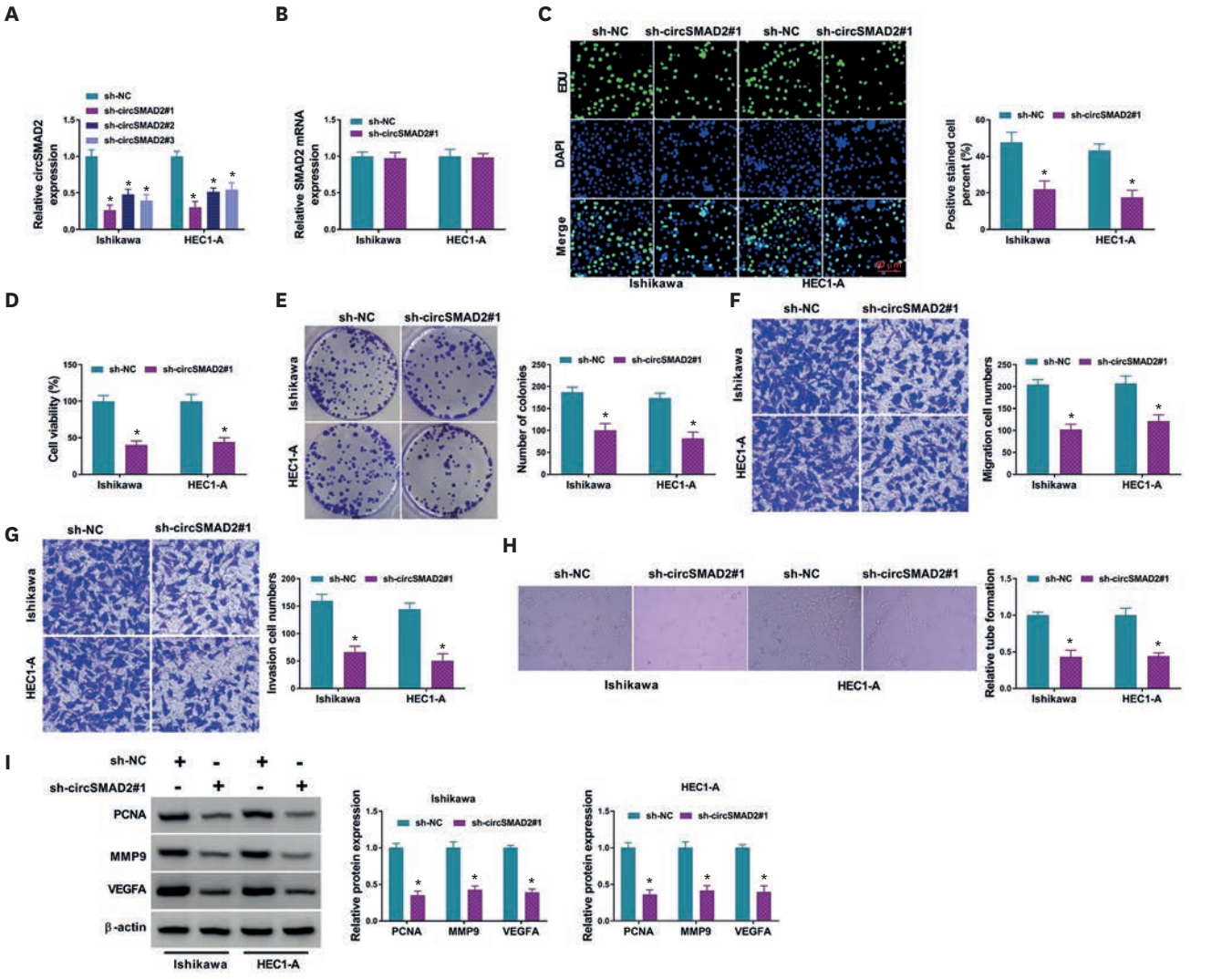

Fig. 1. Knockdown of circSMAD2 inhibits EC cell proliferation, migration, invasion and angiogenesis. (A-B) quantitative real-time polymerase chain reaction analysis of circSMAD2 and SMAD2 mRNA expression in Ishikawa and HEC1-A cells transfected with sh-circSMAD2. (C-I) sh-NC or sh-circSMAD2#1 was transfected into Ishikawa and HEC1-A cells. (C-D) EDU and CCK-8 assay were applied to detect cell proliferation and viability. (E) Colony formation assay was conducted to measure detect cell colony-forming ability. (F-G) Transwell migration and invasion assays were adopted to evaluate the migration and invasion abilities of Ishikawa and HEC1-A cells. (H) Angiogenesis capability was assessed by tube formation assay. (I) The expression of PCNA, MMP9 and VEGFA were examined using Western blot.
*p<0.001.

results showed that miR-1277-5p mimic was confirmed to increase miR-1277-5p expression in Ishikawa and HEC1-A cells (**Fig. 2C**). MiR-1277-5p mimic could reduce the luciferase activity of circSMAD2-WT vector, while had no effect on that of the circSMAD2-MUT vector (**Fig. 2D**). In addition, circSMAD2 and miR-1277-5p could be enriched by anti-AGO2, and Bio-miR-1277-5p probe could enrich more circSMAD2 compared to Bio-NC (**Fig. 2E and F**). Next, miR-1277-5p was downregulated in EC tissues and cells (**Fig. 2G and H**). Moreover, after transfected with sh-circSMAD2#1, the miR-1277-5p expression in Ishikawa and HEC1-A cells was increased (**Fig. 2I**).

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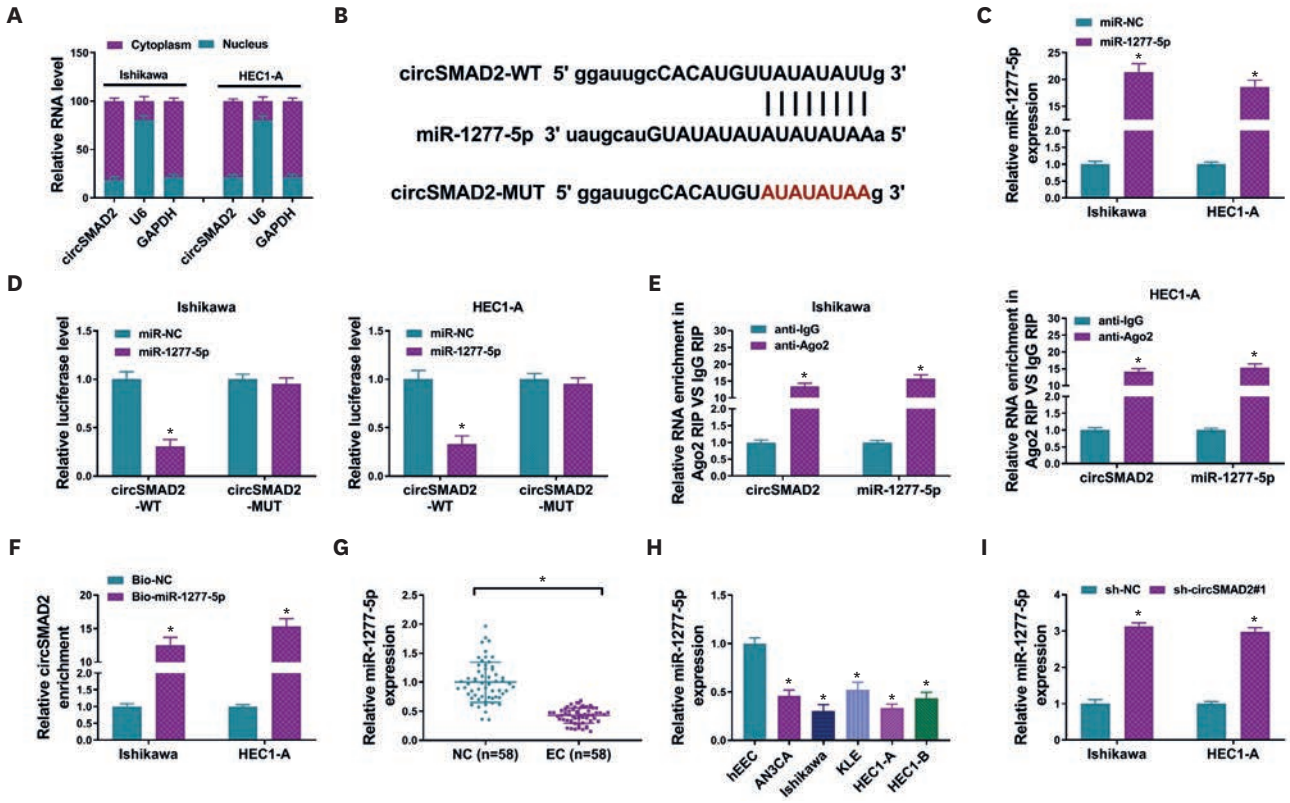

Fig. 2. Circ SMAD2 acts as a sponge for miR-1277-5p in EC cells. (A) Subcellular localization of circSMAD2 in EC cells was analyzed by qRT-PCR. (B) The binding site between circSMAD2 and miR-1277-5p was predicted by starbase online database. (C) qRT-PCR was used to detect the expression of miR-1277-5p in Ishikawa and HEC1-A cells transfected miR-NC or miR-1277-5p. (D) Luciferase activity assays were performed in Ishikawa and HEC1-A cells co-transfected with reporter plasmid (circSMAD2-WT or circSMAD2-MUT) and the indicated miRNAs (miR-NC or miR-1277-5p). (E-F) RNA pull-down and RIP assays were applied to detect the relationship between circSMAD2 and miR-1277-5p. (G) miR-1277-5p expression in EC tissues (n=58) and normal tissues (n=58) was analyzed by qRT-PCR (H) The expression of miR-1277-5p was examined in different cells. (I) The relative levels of miR-1277-5p in Ishikawa and HEC1-A cell lines transfected with sh-circSMAD2#1 or sh-NC were detected by qRT-PCR. EC, endometrial cancer; MUT, mutant; qRT-PCR, quantitative real-time polymerase chain reaction; WT, wild type.
*p<0.001.

3. Silencing miR-1277-5p reversed the sh-circSMAD2-induced antitumor effects in EC cells

Several miRNA rescue experiments was applied to investigate whether miR-1277-5p could reverse the sh-circSMAD2 effect in EC cells. Firstly, miR-1277-5p inhibitor could reduce circSMAD2 silencing-induced elevation of miR-1277-5 expression (**Fig. 3A**). miR-1277-5p inhibitor reversed circSMAD2 knockdown-induced inhibition in cell viability and colony numbers (**Fig. 3B-D**). Knockdown of both miR-1277-5p could partly rescue the loss of motility abilities in sh-circSMAD2-transfected EC cell lines (**Fig. 3E and F**). Additionally, the inhibitory effect of sh-circSMAD2 on cell angiogenesis capability was reversed after miR-1277-5p inhibitor co-transfection (**Fig. 3G**). Consistently, we found that miR-1277-5p inhibitor reversed circSMAD2 knockdown-reduced PCNA, MMP9 and VEGFA expression (**Fig. 3H**).

4. MFGE8 was a direct target of miR-1277-5p

MFGE8 was predicted as a putative target gene of miR-1277-5p (**Fig. 4A**). miR-1277-5p mimic only could reduce the luciferase activity of MFGE8-3'UTR-WT vector but that of the corresponding MUT vector (**Fig. 4B**). Additionally, the protein level of MFGE8 was significantly decreased by miR-145-5p mimics but was increased by miR-1277-5p inhibitor (**Fig. 4C**). Further, sh-circSMAD2-induced inhibition in MFGE8 expression was inversely regulated by miR-1277-5p inhibitor (**Fig. 4D**).

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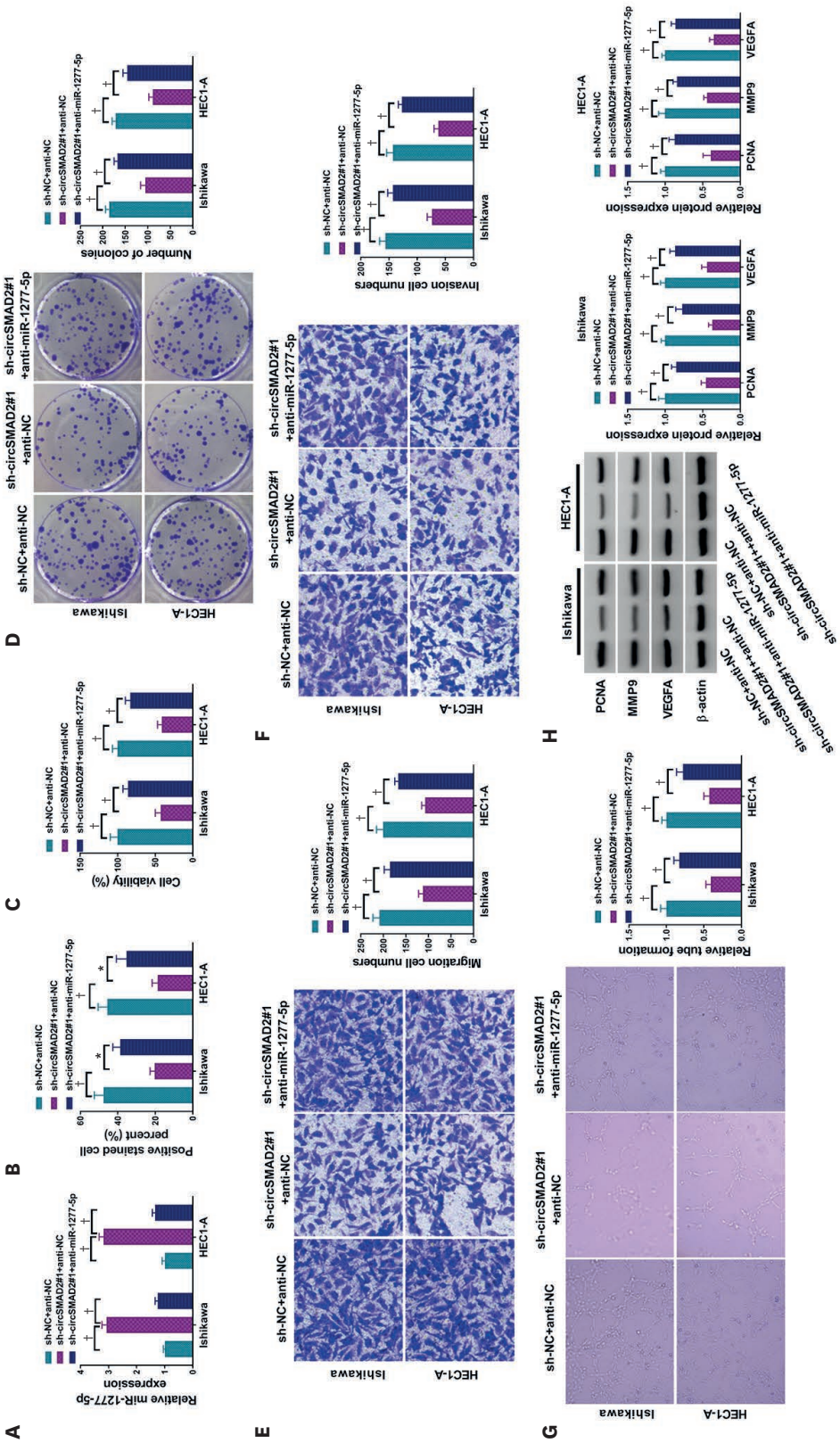

Fig. 3. The regulation of circSMAD2 on endometrial cancer cells is mediated by miR-1277-5p. (A) The inhibition efficiency of anti-miR-1277-5p was confirmed by measuring miR-1277-5p expression using quantitative real-time polymerase chain reaction. (B-H) Ishikawa and HEC1-A cells were transfected with sh-NC + anti-NC, sh-circSMAD2#1 + anti-NC or sh-circSMAD2#1 + anti-miR-1277-5p. EDU assay (B), CCK8 assay (C), colony formation assay (D), transwell assay (E-F) and tube formation assay (G) were utilized to examine cell proliferation, cell viability, colony-forming ability, the numbers of migrated and invaded cells, and angiogenesis capability. (H) Western blot analysis was used to determine the protein levels of PCNA, MMP9 and VEGFA.
MUT, mutant; WT, wild type.
*p<0.001.

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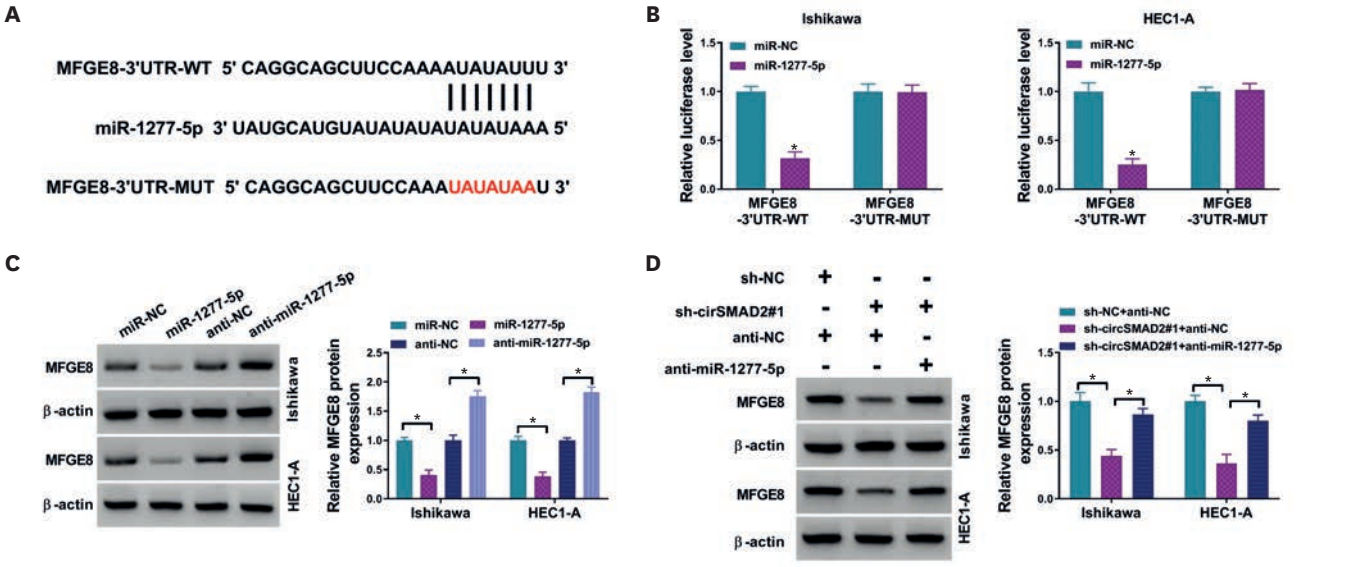

Fig. 4. MFGE8 is a direct target of miR-1277-5p in endometrial cancer cells. (A) The predicted binding sites between MFGE8-3'UTR and miR-1277-5p was predicted by Targetscan online database. (B) The dual-luciferase reporter experiment showed that miR-1277-5p remarkably reduced the activity of wild-type MFGE8-3'UTR reporter. (C) Western blot analysis was employed to determine the effects of miR-1277-5p mimics and inhibitors on MFGE8 protein expression in Ishikawa and HEC1-A cells. (D) MFGE8 protein expression was detected in Ishikawa and HEC1-A cells transfected with sh-NC + anti-NC, sh-circSMAD2#1 + anti-NC or sh-circSMAD2#1 + anti-miR-1277-5p.
MUT, mutant; WT, wild type.
*p<0.001.

5. Overexpression of MFGE8 reverses the effect of miR-1277-5p on EC cells

Firstly, we confirmed that the expression of MFGE8 was regained after MFGE8 overexpression vector co-transfection (**Fig. 5A**). The inhibitory effect of miR-1277-5p overexpression on EC cell proliferation, cell viability and colony-forming ability could be reversed by overexpressing MFGE8 (**Fig. 5B-D**). Moreover, overexpression of MFGE8 countervailed the suppressive effect of miR-1277-5p overexpression on cell migration, invasion and angiogenesis capability (**Fig. 5E-G**). Meanwhile, the negative regulation of miR-1277-5p overexpression on PCNA, MMP9 and VEGFA expression was recovered by upregulating MFGE8 (**Fig. 5H**). Additionally, MFGE8 was upregulated in EC tissues compared with matched adjacent normal tissues (**Fig. S2A and B**). Meanwhile, the **Fig. S2C** results suggested MFGE8 was upregulated in EC cell lines. Then, we transfected si-MFGE8 into Ishikawa and HEC1-A cells to knock down MFGE8 expression (**Fig. S2D**). Consistently, function-based experiments were performed. Decreased expression of MFGE8 significantly inhibited the cell proliferation, cell viability, cell migration, cell invasion, and angiogenesis capability compared with control cells in Ishikawa and HEC1-A cells (**Fig. S2E-J**). Consistently, knockdown of MFGE8 also decreased PCNA, MMP9 and VEGFA protein expression (**Fig. S2K**).

6. Down-regulation of circSMAD2 repressed the growth of EC cells in vivo

Ishikawa cells transfected with sh-circSMAD2#1 or sh-NC were injected with into nude mice. The results showed that silencing circSMAD2 reduced the tumor volume and weight compared to the control group (**Fig. 6A and B**). IHC assays demonstrated that konwdown of circSMAD2 decreased the expression of Ki-67 and MFGE8 in xenograft tumor tissues (**Fig. 6C**). The qRT-PCR results presented that circSMAD2 and MFGE8 expression was decreased and miR-1277-5p expression was increased by circSMAD2 knockdown (**Fig. 6D**). Also, the protein levels of MFGE8, PCNA, MMP9 and VEGFA were reduced in the tumor tissues of the sh-circSMAD2#1 group (**Fig. 6E**).

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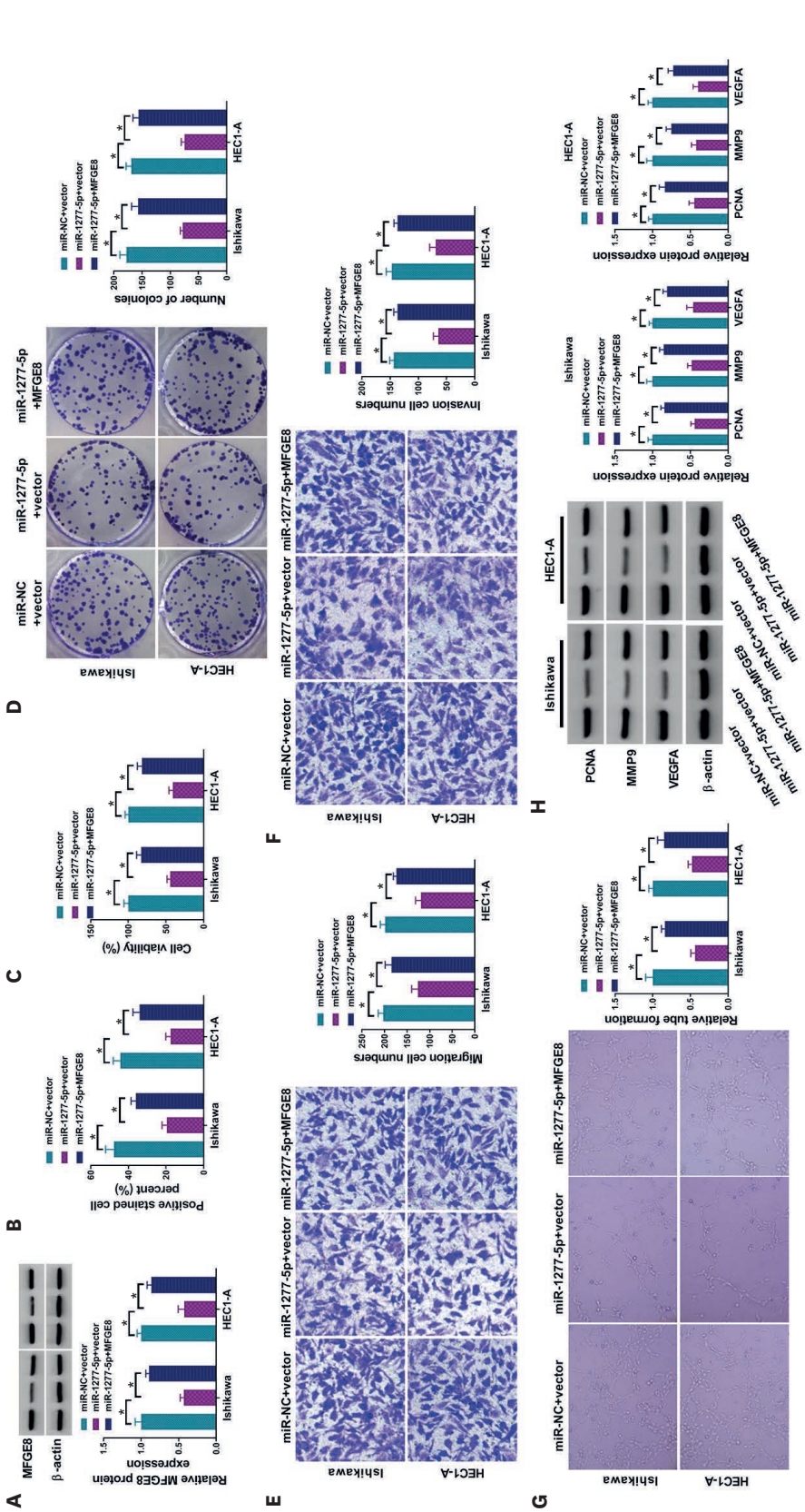

Fig. 5. Overexpression of MFGE8 reverses miR-1277-5p-induced attenuation of proliferation, migration, invasion and angiogenesis in endometrial cancer cells. (A) The overexpression efficiency of MFGE8 was examined by measuring MFGE protein expression using Western blot. (B-H) Ishikawa and HEC1-A cells were transfected with miR-NC + vector, miR-1277-5p + vector or miR-1277-5p + MFGE8. EDU assay (B), CCK8 assay (C), and colony formation assay (D) were used to detect cell proliferation, cell viability, colony-forming ability. Transwell assay (E-F) and tube formation assay (G) were utilized to monitor cell's migrated and invaded ability and angiogenesis capability. (H) The protein levels of PCNA, MMP9 and VEGFA were evaluated using Western blot analysis.

*$p<0.001$.

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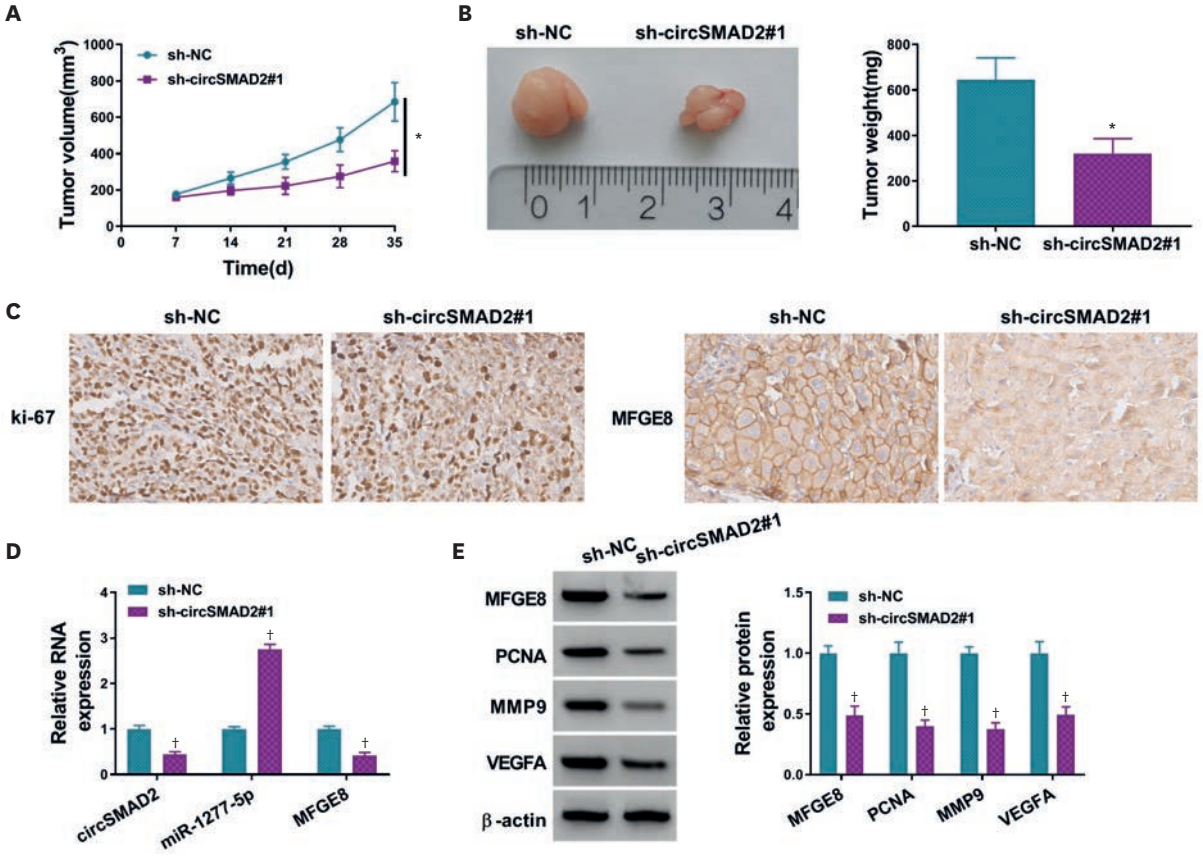

Fig. 6. circSMAD2 knockdown inhibits the growth of endometrial cancer cells in vivo. Ishikawa cells transfected with sh-circSMAD2#1 or sh-NC were injected into nude mice. (A) The tumor volume curve of nude mice was analyzed. (B) The tumor weights of nude mice were measured. (C) The MFGE8 and Ki-67 positive cells in the tumor tissues of mice were assessed by IHC assay. (D) The expression of circSMAD2, miR-1277-5p, and MFGE8 mRNA was examined by quantitative real-time polymerase chain reaction. (E) The protein expression of MFGE8, PCNA, MMP9 and VEGFA were evaluated using Western blot analysis.
*p<0.01, †p<0.001.

DISCUSSION

Accumulating evidence proves that circRNAs can play crucial functions in human cancer [22]. CircRNA is abnormally expression in gynecologic cancers and play critical roles in tumor progression, including endometrial carcinoma [23,24]. Hsa_circ_0075960 was down-regulated and inhibited EC cell processes through miR-361-3p/SH2B1 pathway [25]. Circ_0109046 might function as a diagnostic maker for EC patients [26]. Liu et al. [27] found that circ_0067835 knockdown blocks cell metastasis in EC through downregulating HMGA1 by targeting miR-342-5p. Jia et al. [28] found that overexpression of hsa_circRNA_0001776 could suppress proliferation and promote apoptosis in EC through miR-182 /LRIG2 signal pathway. Thus, circRNA may be potential targets for EC. In this work, we explored the role of circSMAD2 In EC progression. Consistent with previous results [18], we also corroborated that circSMAD2 was greatly upregulated in EC tissues. In addition, in vitro assays unveiled that silencing of circSMAD2 inhibited EC cell proliferation, metastasis and angiogenesis. Meanwhile, circSMAD2 depletion constrained EC growth in vivo.

CircRNAs can indirectly regulate the target genes by sponging miRNAs [29,30]. CircRNA circDDX17 inhibited breast cancer cell growth and invasion through miR-605 [31]. Circ_0007142 functioned as a competing circRNA of miR-103a-2-5p in colorectal cancer

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[32]. The expression levels of HMGA2 was elevated by upregulating circ_100565 in lung cancer [33]. CircFLNA elevated the expression of FLNA through targeting miR-486-3p induce laryngeal squamous cell carcinoma migration and invasion [34]. Bioinformatic analysis predicted that circSMAD2 has binding sites for miRNA-1277-5p. MiR-1277-5p plays important roles in tumor progression. For example, HOTAIR accelerated the malignant progression of gastric cancer through upregulating COL5A1 by targeting miR-1277-5p [35]. The increased expression of miR-1277-5p curbed growth and migration of HepG2 cells [36]. But, there were no study that reported the mechanism of miR-1277-5p in EC progression. In this work, we identified circSMAD2 bound to miR-1277-5p. MiR-1277-5p expression was decreased in EC tissues and cells. Furthermore, we found that the effect of circSMAD2 knockdown on EC cells progression was reversed by miR-1277-5p inhibitors.

MFGE8 is a glycoprotein of 46 kDa and is involved in tumour progression. For example, MFG-E8 was overexpressed in epithelial ovarian cancer [37]. Wu et al. [38] showed that knockdown of MFG-E8 may hinder the immunosuppressive microenvironment of glioma, thereby improving tumor progression. In addition, MFGE8 has the potential as a diagnostic or prognostic marker for cancer [39,40]. The present work was the first one to reveal the function of MFGE8 in EC. Our study showed that MFGE8 was robustly overexpressed in EC tissues and cells. MiR-1277-5p could directly bind to MFGE8 through binding with its 3'UTR sites. In addition, overexpression of MFGE8 could rescue miR-1277-5p elevation-mediated on EC cell malignant behaviors. Furthermore, circSMAD2 could promote MFGE8 production through miR-1277-5p Ishikawa and HEC1-A cells. Thus, circSMAD2 regulated EC malignant progression by the miR-1277-5p/MFGE8 axis. However, the mechanism of MFGE8 regulating EC cell malignancy has not been analyzed in this study. Further assay needs to be performed to address this issue.

In conclusion, we found circSMAD2 could function as an oncogene in EC and promote the progression in EC cells. The circSMAD2/miR-1277-5p/MFGE8 axis might be a potential therapeutic for EC patients treatment.

SUPPLEMENTARY MATERIALS

Table S1

Primer sequences used for quantitative real-time polymerase chain reaction

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Fig. S1

circSMAD2 is overexpressed in EC tissues and cells. (A) circSMAD2 expression in EC tissues (n=58) and adjacent normal tissues (n=58) was analyzed by qRT-PCR; (B) qRT-PCR was used to detect circSMAD2 expression level in human normal endometrial epithelial cells and EC cell lines; (C) Kaplan–Meier survival analysis was implemented to assess the relationship between circSMAD2 expression and OS in EC patients (n=58); (D) The expressions of linear and circular RNAs in cells received RNase R or mock treatment were analyzed. All experiments were performed in triplicate.

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Fig. S2

MFGE8 regulates EC cell proliferation, migration, invasion and angiogenesis. (A-C) MFGE8 expression was determined by quantitative real-time polymerase chain reaction and Western blot in EC tissues and cells. (D) The inhibition efficiency of MFGE8 was confirmed by Western blot. After Ishikawa and HEC1-A cells were treated with si-NC, si-MFGE8. (E-K) si-NC or si-MFGE8 was transfected into Ishikawa and HEC1-A cells. Cell proliferation (E), cell viability (F), colony number (G), cell migration and invasion (H-I), angiogenesis capability (J) and related protein levels (K) were measured to by EDU assay, CCK8 assay, colony formation assay, transwell assay, tube formation assay and western blot, respectively.

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