Estrogen receptor beta increases clear cell renal cell carcinoma stem cell phenotype via altering the circPHACTR4/miR-34b-5p/c-Myc signaling

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Funding information
This study was supported by the National Natural Science Foundation of China (grant numbers 81802544, 82072842, 81970216); the Natural Science Foundation of Hebei Province (grant number H2020206146); and supported by the Excellent Youth Science Foundation of Hebei Province (grant number H2019206536).

Abstract
Early clinical studies indicated that estrogen receptor beta (ERβ) might play key roles to impact the progression of clear cell renal cell carcinoma (ccRCC). The detailed molecular mechanisms, however, remain unclear. Here, we found ERβ could increase the cancer stem cell (CSC) population via altering the circPHACTR4/miR-34b-5p/c-Myc signaling. Mechanism dissection revealed that ERβ could suppress circular RNA PHACTR4 (circPHACTR4) expression via direct binding to the estrogen response elements (EREs) on the 5’ promoter region of its host gene, phosphatase and actin regulator 4 (PHACTR4) to decrease miR-34b-5p expression. The decreased miRNA-34b-5p could then increase c-Myc mRNA translation via targeting its 3’ untranslated region (3’ UTR). The in vivo mouse model with subcutaneous xenografts of ccRCC cells also validated the in vitro data. Importantly, analysis results from ccRCC TCGA database and our clinical data further confirmed the above in vitro/in vivo data. Together, these results suggest that ERβ may increase CSC population in ccRCC via altering ERβ/circPHACTR4/miR-34b-5p/c-Myc signaling and that targeting this newly identified signal pathway may help physicians to better suppress ccRCC progression.

KEYWORDS
circular RNA, CSCs, estrogen receptor beta, miRNA, renal cell carcinoma

Abbreviations: 3’ UTR, 3’ untranslated region; ccRCC, clear cell renal cell carcinoma; ChIP, chromatin immunoprecipitation; circPHACTR4, circular RNA PHACTR4; circRNAs, circular RNAs; CSCs, cancer stem cells; EREs, estrogen response elements; ERβ, estrogen receptor beta; FISH, fluorescence in situ hybridization; miRNA, microRNA; PCR, polymerase chain reaction; PHACTR4, phosphatase and actin regulator 4; TCGA, the cancer genome atlas.

Yaxuan Wang and Zhan Yang contributed equally to this work.

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1 | INTRODUCTION

The incidence of renal cell carcinoma (RCC) is increasing yearly. RCC has been the sixth most common cancer in men and the eighth most common in women worldwide, with more than 140,000 deaths annually. The most common histologic subtype of RCC is clear cell RCC (ccRCC), accounting for about 70%–80%, which is the most malignant subtype among other RCC subtypes. With the development of diagnostic technology, more and more small ccRCC are diagnosed and treated with partial or radical nephrectomy. However, approximately 20%–30% of the ccRCC patients still progress to metastatic lesions, which indicates a poor 5-year survival rate of less than 10%.

Cancer stem cells (CSCs) were first introduced 20 years ago. CSCs are a small subpopulation of cells within tumors, which exhibit enhanced capabilities of self-renewal, clonogenicity, and tumor initiation. They are considered as seeds for tumor occurrence, progression, and metastasis. So far, renal cancer CSCs have been successfully isolated. More and more studies reported that CSCs may play an important role in the occurrence, progression, drug resistance, recurrence, and metastasis of RCC. Therefore, CSCs may be one of the main causes of therapeutic failure. Eradication of CSCs may be a key factor in improving the prognosis of RCC patients.

There are mainly two estrogen receptor isoforms, ERα and ERβ, which are encoded by genes located in different chromosomal positions and play different cellular roles. Our previous study found that the expression of ERβ in ccRCC was much higher than that of ERα. Previously, ERβ was thought to cause cell senescence or death in breast cancer and prostate cancer. However, the function of ERβ was different in various cancer types. ERβ can promote progression in bladder cancer, glioblastomas, lung cancer, and kidney cancer. It has been reported that ERβ may play an important role in CSCs. Ma and colleagues reported that ERβ can promote CSCs in breast cancer. Li and colleagues reported that ERβ/IncRNA-H19 positive feedback loop can promote CSCs in papillary thyroid cancer under E2 treatment. However, the molecular mechanism of ERβ regulation of CSCs in ccRCC remains poorly understood.

Circular RNAs (circRNAs) are a class of noncoding endogenous RNAs, a large number of which are endogenous, conserved, stable, and specific in eukaryotic cells. CircRNAs are generally considered to be produced by circularization of exons with or without introns through nonspecific mechanisms. It is generally believed that the function of circRNA is acting as miRNA sponge or miRNA reservoir via binding sites. Emerging studies have revealed that circRNAs with an intron sequence could regulate transcription, and circRNAs could also encode peptides. Recently, it was reported that circRNAs could regulate CSCs for cancer progression. Our new finding indicated that ERβ could regulate circRNA to promote CSC properties in ccRCC. However, how circRNAs promote the cancer initiation and progression via CSCs remains to be elucidated.

Here, we reported that ERβ could function through regulating the circRNA PHACTR4 (circPHACTR4) and controlling of miR-34b-5p/c-Myc to promote CSCs properties in ccRCC.

2 | MATERIALS AND METHODS

2.1 | Human ccRCC specimens

This study recruited 10 kidney cancer patients who underwent radical nephrectomy from the Department of Urology, the Second Hospital of Hebei Medical University. All of the patients did not receive any preoperative treatment, and all of their postoperative pathology was confirmed as clear cell renal cell carcinoma (ccRCC). Two different types of tissues from each ccRCC patient were processed immediately after surgical resection, including nontumor tissue (N) > 2 cm far from the tumor edge, and tissue from the tumor (T). The specimens from each patient were processed in two methods. One method was snap-frozen immediately after resection and stored in liquid nitrogen for further experiments. The other method was fixed with 10% formaldehyde solution and paraffin embedded for further experiments.

2.2 | Reagents and materials

GAPDH, β-actin, ERβ, c-Myc antibodies, and normal rabbit IgG were purchased from Proteintech (China). Antibase/rabbit antibody for Western Blot was from KPL (USA).

2.3 | In vitro cell culture/maintenance

The 786-O, A498, and HEK-293 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). All the cells were authenticated by short tandem repeat (STR) DNA profiling, and were tested to be mycoplasma free using the Universal Mycoplasma Detection Kit (ATCC) yearly. All cell lines were cultured in Dulbecco’s Modified Eagle’s (DMEM) media (Invitrogen, Grand Island, NY, USA) supplemented with penicillin (25 units/ml), 10% fetal bovine serum...
(FBS) and streptomycin (25 g/ml), and 1% L-glutamine. All cell lines were cultured in a 5% (v/v) CO2 humidified incubator at 37°C.

2.4 | Lentiviral expression plasmid construction and virus production

According to Addgene’s protocol, the pLKO.1-shERβ, pWP-oeERβ (Plasmid #12254), pLVTHM-shcircPHACTR4 (Plasmid #12247), pWP-oeircPHACTR4, pLVTHM-oemiR-34b-5p, pLVTHM-shc-Myc, psPAX2 packaging plasmid, and pMD2G envelope plasmid, were then transfected into HEK-293 cells using the standard calcium chloride transfection method for 48 h to get the lentivirus soup. The lentivirus soups were collected and concentrated by density gradient centrifugation, then frozen in −80°C for use.

2.5 | The circPHACTR4 overexpression plasmid construction

The pWP-oeircPHACTR4 was constructed by PCR amplifying the circRNA locus, including 200 bp downstream and 1000 bp upstream of the nonlinear splice sites, using circPHACTR4 forward/reverse primers, respectively. The PCR fragment was inserted into pWP1 (Invitrogen).

2.6 | RNA extraction and quantitative real-time PCR analysis

Trizol reagent (Invitrogen, Grand Island, NY) was used to isolate RNAs. RNA was used for reverse transcription using Superscript III transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was applied using a Bio-Rad CFX96 system with SYBR green to determine the mRNA expression level of the genes of interest. Expression levels were normalized to the expression of β-actin and/or GAPDH. The qRT-PCR protocol was as follows: 50°C for 2 min, 95°C for 8 min 30 s, followed by 45 cycles at 95°C for 15 s, and 60°C for 1 min. The extension was 95°C for 1 min, 55°C for 1 min, and 55°C for 10 s. GAPDH was used as a normalized control. The miRNAs were extracted using PureLink® miRNA kit. Briefly, 2 μg RNA was used for poly A polymerase at 37°C for 20 min, and then we conducted reverse transcriptase by annealing at 65°C for 5 min, and 4°C for 2 min after adding 50 μM RT anchor primer. The last step was cDNA synthesis at 42°C for 60 min by adding 2 μl 10 mM dNTP, 2 μl 5XRT buffer, 1 μl reverse transcriptase, and ddH2O to a total of 20 μl. The qRT-PCR protocol was as follows: 95°C 2 min, followed by 45 cycles at 95°C 15 s and 60°C 45 s. U6 and/or 5S were used as normalized controls.

2.7 | Western blot analysis

Cells were lysed in RIPA buffer and proteins (30 μg) were separated on 8%–12% SDS/PAGE gel and then transferred onto PVDF membranes (Millipore, Billerica, MA). After blocking membranes, they were incubated with appropriate dilutions of specific primary antibodies. The blots were treated with the Immobilon™ Western (Millipore), and detected by ECL (enhanced chemiluminescence) Fuzon Fx (Vilber Lourmat). Images were captured and processed by FusionCapt Advance Fx5 software (Vilber Lourmat).

2.8 | Sphere formation assay

The sphere formation assay was performed as described earlier.34,35 Briefly, single-cell suspensions (1 × 10³, in 70 μl media) were mixed with 70 μl Matrigel (BD, Franklin Lakes) and placed along the rim of the 24-well plates with three triplicate experiments. The culture plates were placed in 37°C incubator for 10 min to let the mixture solidify and 500 μl media was then added into the well. After 7–14 days of incubation, colonies in five random fields per each well were counted under a microscope to evaluate the CSC numbers and size differences were also examined.

2.9 | MTT proliferation assay

MTT proliferation assay was used to measure cell proliferation rates. 786-O cells were seeded in 24-well plate at 5000 cells per well after being transfected with specific plasmids for 24 h. The cell proliferation assay was performed on day 1 to day 7. We first added MTT reagent to each well, then incubated at 37°C for 2 h, dissolved the precipitate in DMSO and measured the absorbance at 450 nm.

2.10 | Colony formation assay

786-O cells were seeded in 6-well plates at 2000 cells per well in growth media after being transfected with specific plasmids for 24 h. After incubating for 7 to 14 days, the colonies were washed with PBS, fixed with 10% formalin, and stained with 0.5% crystal violet. After digital photography, the total colony area was counted under Olympus light microscope.
2.11 Chromatin immunoprecipitation assay

Cells were cross-linked with 4% formaldehyde followed by cell collection and sonication with a predetermined power. Lysates were precleared sequentially with normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) and protein A-agarose. Anti-ERβ antibody (2.0 µg) was added to the cell lysates and incubated overnight at 4°C. IgG was used in the reaction for negative control.

![Figure 1](image1.png)

**Figure 1** ERβ increases the CSC formation in ccRCC. (A) Western blot verification of ERβ knockdown in 786-O cells (left panel) and overexpression in A498 cells (right panel). (B) Sphere formation assays were performed using 786-O cells (transfected with ERβ-shRNA and pLKO, upper panel) and A498 cells (transfected with ERβ-cDNA and pWPI, lower panel) to evaluate the CSCs number. After 7–14 days of incubation, colonies in five random fields per each well were counted under a microscope. Quantitations for B are shown at the right and data presented as mean ± SD. **p < .01 compared to the controls, Scale bar: 500 μm.

![Figure 2](image2.png)

**Figure 2** ERβ increases the CSC formation in ccRCC cells via altering circPHACTR4 expression. (A) The qRT-PCR assay for screening a group of ccRCC associated circRNAs in 786-O cells with knocked down ERβ (shERβ) compared with vector (pLKO) and in A498 cells with overexpressed ERβ (oeERβ) compared with vector (pWPI). (B) Sanger sequencing confirmed head-to-tail splicing of circPHACTR4. (C) The circPHACTR4 is resistant to RNase R treatment. (D) The knockdown and overexpression efficiency were identified by qRT-PCR in A498 cells (shcircPHACTR4 vs. pLVTHM, left panel) and in 786-O cells (ocircPHACTR4 vs. pWPI, right panel), respectively. (E) RNA fluorescence in situ hybridization for the detection of circPHACTR4, showing the localization of circPHACTR4 (detected with a junction probe). (F) Sphere formation assay were performed to verify the roles of ERβ and circPHACTR4 using 786-O cells transfected with pLKO+pLVTHM, shERβ+pLVTHM, pLKO+shcircPHACTR4, or shERβ+shcircPHACTR4. (G) Sphere formation assay were performed using A498 cells transfected with pWPI+pWPI, oeERβ+pWPI, pWPI+ocircPHACTR4, or oeERβ+ocircPHACTR4. After 7–14 days of incubation, colonies in five random fields per each well were counted under a microscope to evaluate the CSC numbers. Quantitations for F–G are shown at the right and data presented as mean ± SD. *p < .05, **p < .01, ***p < .001 compared to the controls, and Scale bar: 500 μm.
Specific primer sets were designed to amplify a target sequence within the human PHACTR4 promoter and agarose gel electrophoresis was used to identify the PCR products.

### 2.12 Luciferase reporter assays

The human promoter region of PHACTR4 5' promoter was constructed into pGL3-basic vector (Promega, Madison, Wisconsin, USA).
**FIGURE 3** Mechanism dissection how ERβ suppresses circPHACTR4 expression. (A) There are two putative estrogen response elements (EREs) within the 1-Kb promoter region of *PHACTR4*, host gene of circPHACTR4. (B) ChIP products were amplified by PCR reaction. DNA agarose gel electrophoresis of PCR product revealed the ERβ could bind to the potential ERE binding site (−460 to −446 nt). (C) Diagram of cloning the 1-kb *PHACTR4* promoter into pGL3 basic luciferase report vector (pGL3). Site-directed mutagenesis of ERE was changed to BamH1 cutting site (−GGATCC−). (D, E) Co-transfection of wild-type or mutant *PHACTR4* promoter pGL3-Luciferase constructs into 786-O with/without shERβ and A498 cells with/without oeERβ. The luciferase assay was applied to detect the promoter activity. Data are presented as mean ± SD. *p < .05, **p < .01, NS, not significant compared to the controls.
Site-directed mutagenesis of the ERβ binding site in the PHACTR4 5′ promoter was achieved with the Quick-Change mutagenesis. 1000 bp fragments of c-Myc 3′ UTR involving wild type or mutant miRNA-responsive elements were cloned into the psiCHECK2 vector construct (Promega). Luciferase activity was measured by Dual-Luciferase Assay (Promega).

2.13 | Fluorescence in situ hybridization

The cells were fixed in 4% paraformaldehyde for 5 min at room temperature and then washed with PBS. Hybridization with the fluorescence-labeled circPHACTR4 probe in hybridization buffer (Exiqon) was performed during a 1 h incubation at 55°C in a thermoblock (Labnet, Edison, NJ, USA). Next, the samples were strictly washed with SSC buffer and then treated with DAPI (157574, MB biomedical) for nuclear counterstaining. Images were captured using a confocal microscope (DM6000 CFS; Leica) and processed using LAS AF software. Further images were acquired using a Leica DM6000B microscope and digitized using LAS V.4.4 software.

2.14 | RNA pull-down assay

The cells were collected with 1ml cell lysis buffer following the designated 72 h treatments. The quantitated cells were rotated overnight at 4°C after adding 1.5 µl RNase inhibitor and 500 pM antisense oligos. The cells were rotated for 2 h at 4°C after adding 10 µl Streptavidin Agarose beads. Total RNA was extracted by Trizol (Invitrogen) according to the manufacturer’s protocol and subjected to qRT-PCR analysis.

2.15 | In vivo studies

Sixty 4–6-week-old BALB/c female mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and randomly divided into four groups (n = 15 in each group) for injection of 1 × 10^6 786-O cells (mixed with Matrigel, 1:1) under the right forelimb to establish xenograft tumors. No experimental animals were excluded from data (n = 15 for each group). Tumor weights and lengths were measured and calculated. The study protocol was approved by the Ethics Committee of the Second Hospital of Hebei Medical University.

2.16 | Immunofluorescence staining

In brief, 5-µm paraffin-embedded cross-sections of tissues were subjected to immunofluorescence staining. Sections were deparaffinized with xylene, rehydrated, and preincubated with 10% normal goat serum (710027, KPL, USA) followed by incubation with the following primary antibodies: anti-ERβ (14007-1-AP, Proteintech, China) and anti-c-Myc (10828-1-AP, Proteintech, China). Sections were subsequently treated with the following secondary antibodies: fluorescein-labeled antibody to rabbit IgG (021516, KPL, USA) and rhodamine-labeled antibody to mouse IgG (031806, KPL, USA). In each experiment, DAPI (157574, MB Biomedical) was used for nuclear counterstaining. Images were captured using a confocal microscope (DM6000 CFS, Leica) and processed using LAS AF software.

2.17 | Statistical analysis

Data were expressed as mean ± SD from at least three independent experiments with data points in triplicate. The statistical analysis was performed using Student’s t test or one-way ANOVA with SPSS 22 (IBM Corp, Armonk, NY, USA) or GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). p < .05 was considered statistically significant.

3 | RESULTS

3.1 | ERβ increased the CSC population in ccRCC cells

Recent studies indicated that ERβ could function as an oncogene via regulating the circATP2B1 to promote the ccRCC cell invasion. Here, we found that ERβ could also regulate the CSC population to impact the ccRCC progression. We first detected the ERβ expression levels of renal epithelial HK-2 cells and various ccRCC cell lines (OSRC-2, 786-O, ACHN, 769-P, A498, and Caki-1). ERβ was significantly higher in ccRCC cell lines at the RNA level (Supporting Information Figure S1A) and the protein level (Supporting Information Figure S1B). To further confirm the knockdown specificity and effect, we constructed two ERβ-shRNA (shERβ#1 and shERβ#2). Western blot assays validated that both shERβ#1 and shERβ#2 could effectively knock down ERβ expression in two different ccRCC cell lines (786-O and Caki-1) (Supporting Information Figure S1C). Next, the qRT-PCR assays validated that both shERβ#1 and shERβ#2 could
effectively knock down ERβ expression in 786-O cells, and oeERβ could significantly overexpress the ERβ level in A498 cells (Supporting Information Figure S1D). We then knocked down ERβ (shERβ) in 786-O ccRCC cells that have a higher endogenous ERβ expression, and overexpressed ERβ (oeERβ) in A498 cells that have a lower
endogenous ERβ expression (Figure 1A). At the same time, we verified the ERβ antibody in HEK-293 cells by knocked down and overexpression of ERβ (Supporting Information Figure S1E). Results from sphere formation assays revealed that knocking down ERβ in 786-O cells (786-O-shERβ) led to decrease the CSC population (Figure 1B, upper panels). In contrast, overexpressing ERβ in A498 cells (A498-oeERβ) led to increase the CSC population (Figure 1B, lower panels). In addition, results from CSC-related biomarkers also revealed that knocking down ERβ in 786-O cells could decrease the expression of CSC-related biomarkers, while overexpressing ERβ in A498 cells could increase the expression of CSC-related biomarkers, including CD24, CD133, CD105, CD44, NANO-G, and ALDH1A3 (Supporting Information Figure S1F).

Together, results from Figure 1A,B, Supporting Information Figure S1A–F demonstrated that ERβ could increase the CSC population in ccRCC cells.

3.2 ERβ increased the CSC population in ccRCC cells via down-regulating the circPHACTR4

To further dissect the molecular mechanism of how ERβ can increase the CSC population in ccRCC cells, we focused on circRNAs since an early study indicated that ERβ might impact the ccRCC progression via altering the circATP2B1 expression. Results from bioinformatics analysis of circ2Traits36 revealed 76 ccRCC related circRNAs, and qRT-PCR data further found that circATP2B1 (hsa_circ_000826) was regulated by ERβ. Interestingly, we also found that knocking down ERβ in 786-O cells led to decrease the expression of hsa_circ_001953, and increase the expression of hsa_circ_000826 and hsa_circ_000570 (Figure 2A, left panel). Importantly, we found that overexpressing ERβ in A498 cells also led to decrease the expression of hsa_circ_000826 (circATP2B1) and hsa_circ_000570 (circPHACTR4) (Figure 2A, right panel).

Therefore, we were interested to further study the impact of ERβ-suppressed circPHACTR4 on the ccRCC progression. First, we designed additional divergent primers with nucleotide bases overlapping the junction of circPHACTR4 to identify the circular characteristics. Sanger sequencing revealed the expression of circPHACTR4 (Figure 2B). To further confirm the circular characteristics of the circPHACTR4, we then applied RNase R digestion assay to verify that circPHACTR4 is in the circular form as it is resistant to the RNase R digestion in A498 cells (Figure 2C). Next, we constructed the circPHACTR4 shRNA (shcircPHACTR4) and the overexpressed circPHACTR4 (oecircPHACTR4), and applied the qRT-PCR assays to identify their efficiency in A498 cells and 786-O cells, respectively. (Figure 2D). To better predict the function of the circPHACTR4, we then performed FISH assay with a probe against circPHACTR4 to pinpoint the localization in 786-O cells and A498 cells. The result showed that circPHACTR4 was mainly localized in the cytoplasm (Figure 2E). The results from the rescue experiments via sphere formation assay in 786-O cells (Figure 2F, pLKO+pLVTHM, shERβ+pLVTHM, pLKO+shcircPHACTR4, or shERβ+shcircPHACTR4) and in A498 cells (Figure 2G, pWPI+pWPI, oeERβ+pWPI, pWPI+oecircPHACTR4, or oeERβ+oeircPHACTR4) revealed that knocking down circPHACTR4 could reverse the ERβ-shRNA-decreased CSC population in 786-O cells (Figure 2F). In contrast, overexpressed circPHACTR4 could block/reverse the ERβ-increased CSC population in A498 cells (Figure 2G).

Then, we constructed the second circPHACTR4 shRNA (shcircPHACTR4#2). The qRT-PCR assays validated that both shcircPHACTR4#1 and shcircPHACTR4#2 could effectively knock down circPHACTR4 expression in A498 cells (Supporting Information Figure S2A). Then, we validated the rescue experiments using sphere formation.
assay in 786-O cells with shERβ#2 and shcircPHACTR4#2 (Supporting Information Figure S2B, pLKO+pLVTHM, shERβ#2+pLVTHM, pLKO+shcircPHACTR4#2, or shERβ#2+shcircPHACTR4#2). In addition, the shERβ#2 and shcircPHACTR4#2 were also used to validate whether circPHACTR4 knockdown can reverse the ERβ

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FIGURE 5 Legend on next page
3.3 | Mechanism dissection how ERβ regulates circPHACTR4 expression

Previous studies identified that circRNAs are proportional to their host gene expression. Therefore, we further studied the linkage of ERβ-regulated circPHACTR4 to the host gene, PHACTR4. Through bioinformatic search (Ensembl and PROMO 3.0) of estrogen response elements (EREs) on the 1-kb 5′ promoter region of PHACTR4, we found two potential EREs within 1-kb upstream of the transcriptional start site (Figure 3A). Results from the chromatin immunoprecipitation (ChIP) binding assays in 786-O cells revealed that ERβ could specifically bind to the ERE located on the −460 to −446 nt upstream of the transcription start site of PHACTR4 (Figure 3B). Importantly, we further mutated the key sequences within −459 to −454 nt (Figure 3C), and results from the luciferase reporter assays revealed that knocking down ERβ increased the luciferase reporter activity in 786-O cells with wild-type ERE, but not the mutant ERE (Figure 3D). An opposite approach also found that overexpressing ERβ decreased the luciferase reporter activity in A498 cells with wild-type ERE, but not the mutant ERE (Figure 3E).

Together, results from Figure 3A–E demonstrated that ERβ could transcriptionally regulate circPHACTR4 expression via direct binding to the ERE (−460 to −446 nt) on the 5′ promoter of its host gene PHACTR4 in ccRCC cells.

3.4 | ERβ increased the CSC population in ccRCC cells via altering the circPHACTR4/miR-34b-5p

Next, to further study how ERβ-regulated circPHACTR4 can alter the CSC population, we focused on the miRNAs, as early studies indicated that circRNAs might regulate their target genes mainly by sequestering miRNAs. Results from two public bioinformatic prediction databases (CircNet and StarBase) searches for miRNAs linked to circPHACTR4 revealed three potential miRNAs (miR-34b-5p, miR-370-3p, and miR-181b-3p). Further studies showed overexpressing circPHACTR4 in 786-O cells significantly increased the expression of only miR-34b-5p, and knocking down circPHACTR4 in A498 cells significantly decreased the expression of both miR-34b-5p and miR-370-3p (Figure 4A). However, knocking down ERβ in 786-O cells significantly increased the expression of only miR-34b-5p and overexpressing ERβ in A498 cells significantly decreased the expression of miR-34b-5p and significantly increased the expression of miR-181b-3p.
Together, the above consistent results of qRT-PCR indicated that miR-34b-5p expression could be modulated by ERβ/circPHACTR4. We then applied the RNA pull-down assay using the biotinylated oligonucleotides probe to confirm the interaction between circPHACTR4 with these miRNAs, and

![Graphs and scatter plots](image-url)
results revealed that only miR-34b-5p was enriched in the pull-down product (Figure 4C), suggesting the direct binding of miR-34b-5p with circPHACTR4.

Finally, results from the interruption approach further revealed that ERβ-shRNA-increased miR-34b-5p expression in 786-O cells could be blocked/reversed by knocking down circPHACTR4 (Figure 4D), and overexpressing circPHACTR4 in A498 cells also reversed the ERβ-suppressed miR-34b-5p expression (Figure 4E).

These results suggested that circPHACTR4 may not act as a sponge to sequester miRNAs, but function as a “reservoir” to stabilize the expression of miRNAs.20,26,28 To further confirm this possibility, we tested the miR-34b-5p degradation rate at different time points by qRT-PCT. Under the condition of knocking down ERβ in 786-O cells, transducing circPHACTR4-shRNA could lead to a significant reduction of the remaining miR-34b-5p while treating with Actinomycin D to stop new RNA synthesis (Figure 4F).

Next, results from the rescue approaches using sphere formation assays revealed that miR-34b-5p inhibitor partially reversed the ERβ-shRNA-reduced CSC population in 786-O cells (Figure 4G). In contrast, overexpressing miR-34b-5p blocked/reversed the oeERβ-increased CSC population in A498 cells (Figure 4H).

Together, results from Figure 4A–H suggested that ERβ-regulated circPHACTR4 might function via altering the miR-34b-5p expression to impact the CSC population in ccRCC.

3.5 | ERβ/circPHACTR4/miR-34b-5p signaling increases the CSC population in ccRCC cells via altering c-Myc expression

To further dissect how the ERβ/circPHACTR4/miR-34b-5p signaling can increase the CSC population in ccRCC cells, we focused on several key genes (c-Myc, SOX2, CD133, CD24, NANO G, CD105, ALDH1A3, and PAX2) related to the CSCs.10,11 The qRT-PCT results revealed that knocking down ERβ in 786-O cells significantly decreased the expression of c-Myc, CD133, CD24, NANO G, and CD105, while, overexpressing ERβ in A498 cells significantly increased the expression of c-Myc and NANO G (Figure 5A). Then, we screened those genes in 786-O cells with overexpressed circPHACTR4 and we found that the expression of c-Myc, SOX2, CD133, CD24, and NANO G decreased significantly. We also screened those genes in A498 cells with knockdown of circPHACTR4 revealing the knocking increased the c-Myc, SOX2, and CD24 expressions (Figure 5B). Since these genes are CSC markers, their expression level changes can be consistent with the changes of CSC phenotype. However, c-Myc expression showed consistent and significant changes. In addition, bioinformatics analyses (circBase, CircNet, and OncoLnc) indicated that c-Myc could be the target gene. Thus, we decided to focus on the c-Myc for further study (Figure 5C).

The c-Myc is one of the most important proto-oncogenes, and plays an important role in the initiation, maintenance, and progression of various cancers.43 We confirmed the phenotype that knocking down ERβ decreased the c-Myc protein expression in 786-O cells and overexpressing ERβ increased the c-Myc protein expression in A498 cells (Figure 5D). We then confirmed that knocking down circPHACTR4 could partially reverse the shERβ-suppressed c-Myc protein expression in 786-O cells, whereas overexpressing circPHACTR4 could partially reverse the oeERβ-increased c-Myc protein expression in A498 cells (Figure 5E). Importantly, we found that miR-34b-5p inhibitor could partially reverse the shERβ-suppressed c-Myc protein expression in 786-O cells, while overexpressing miR-34b-5p could partially reverse the oeERβ-increased c-Myc protein expression in A498 cells (Figure 5F). Next, we applied the interruption approach using c-Myc-shRNA. The rescue experiments via sphere formation assay revealed that shc-Myc could partially reverse the oeERβ-increased CSC population in A498 cells (Figure 5G).

Then, we dissected the mechanism of how miR-34b-5p can modulate c-Myc expression at the molecular level. Through a bioinformatic analysis (http://www.targetscan.org/vert_71/), we identified the potential binding site located on the 3’ UTR of c-Myc-mRNA (Figure 5H). Next, we contracted the psiCHECK2 vector carrying the 3’ UTR of c-Myc-mRNA with wild-type and mutant miRNA-target sites (Figure 5I). The psiCHECK2 luciferase reporter assay results revealed that overexpressing miR-34b-5p could significantly decrease the luciferase reporter activity of wild type rather than the miRNA-binding site mutant in 786-O cells (Figure 5J). Thus, our data suggested that miR-34b-5p

FIGURE 6 Human clinical study linking the ERβ/circPHACTR4/miR-34b-5p/c-Myc signaling axis to the ccRCC progression. (A) Survival curve from OncoLnc indicated that patients with higher levels of ERβ expression had significantly worse overall survival (n = 522, ERβ mRNA levels lower than 50% versus higher than 50%, p < .0001). (B) Survival curve from OncoLnc indicated that patients with higher levels of c-Myc expression had significantly worse overall survival (n = 522, c-Myc mRNA levels lower than 20% vs. higher than 20%, p = .0143). (C) Based on TCGA database, the Pearson correlation analysis showed a positive correlation between ERβ mRNA and c-Myc mRNA (R = .2353; p < .0001). (D) Data of ccRCC patients from TCGA showed that the level of c-Myc mRNA in tumor (T) tissues was significantly higher than that in normal (N) tissues (p < .0001). (E–G) The qRT-PCR data from 10 clinical ccRCC specimens showed that the expression of ERβ in tumor (T) tissues was significantly higher, while the expression of circPHACTR4 and miR-34b-5p in nontumor (N) tissues were significantly higher. Data are presented as mean ± SD. ***p < .001 compared to the controls.
FIGURE 7 Preclinical study using in vivo mouse model to confirm ERβ/circPHACTR4/miR-34b-5p/c-Myc signaling axis in ccRCC progression. 786-O cells were transduced with (1) pLKO+pLVTHM, (2) shERβ+pLVTHM, (3) pLKO+shcircPHACTR4, or (4) shERβ+shcircPHACTR4. Then, 786-O cells were injected subcutaneously in 200 μl PBS/Matrigel (50:50) into the right forelimb of nude mice to establish xenograft tumors (n = 15 in each group). (A) The tumor volumes in each group were measured in situ within four weeks after injection. (B, C) After sacrificing the mice, the resected tumors were measured. (D) The tumor volume was determined by direct measurement with a caliper and calculated by the following formula: volume = [(length × width²)/2]. (E) Immunofluorescence staining was performed to detect the expression of ERβ and c-Myc in xenograft tumor tissues in vivo. Quantitation shown as mean ± SD. *p < .05, **p < .01 compared to the controls, and Scale bar: 20 μm
may directly target the 3′ UTR of c-Myc-mRNA to suppress its protein expression.

Together, results from Figure 5A–J suggested that ERβ/circPHACTR4/miR-34b-5p signaling could increase the CSC population in ccRCC cells via altering the c-Myc signal.

### 3.6 Human clinical study linking the ERβ/circPHACTR4/miR-34b-5p/c-Myc signaling axis to the ccRCC progression

Human clinical data from OncoLnc (http://www.oncolnc.org/) based on the TCGA database was used to evaluate the overall survival of ccRCC patients with varying expressions of ERβ. The results revealed that patients with higher ERβ mRNA expression had significantly worse overall survival ($p < .0001$, Figure 6A), suggesting a promoting role of ERβ in ccRCC progression. In addition, we also evaluated the overall survival of ccRCC patients with varying expressions of c-Myc. The results revealed that patients with higher c-Myc mRNA expression had significantly worse overall survival ($p = .0143$, Figure 6B).

The UCSC Cancer Genome Browser (http://xena.ucsc.edu/) based on the TCGA database was used to detect the relationship of ERβ and c-Myc in ccRCC patients. The results revealed that c-Myc expression was associated with a higher ERβ expression ($R = .2353$, $p < .0001$, Figure 6C). Moreover, c-Myc mRNA levels were significantly higher in tumor tissues than normal tissues ($p < .0001$, Figure 6D), suggesting a promoting role of c-Myc in ccRCC progression.

Then, we used qRT-PCR to detect the expression level of ERβ, circPHACTR4, and miR-34b-5p in 10 human ccRCC samples. The results indicated that ERβ expression level was significantly higher in tumor (T) tissues (Figure 6E), while circPHACTR4 and miR-34b-5p expression levels were significantly higher in nontumor (N) tissues (Figure 6F,G).

Together, results from Figure 6A–G showed that ERβ and c-Myc were positively correlated with the progression of ccRCC, while circPHACTR4 and miR-34b-5p were negatively correlated with ccRCC, which were consistent with the above experimental results using multiple ccRCC cell lines.

### 3.7 In vivo mouse studies confirmed ERβ/circPHACTR4/miR-34b-5p/c-Myc signaling in ccRCC progression

To confirm the in vitro cell lines data in the in vivo, we performed a ccRCC nude mouse model using subcutaneous implanted 786-O tumors to the right forelimb ($1 \times 10^6$ cells). 786-O cells were transduced with: (1) pLKO+pLVTHM, (2) shERβ+pLVTHM, (3) pLKO+shcircPHACTR4, or (4) shERβ+shcircPHACTR4 (4 groups, 15 mice/group). The tumor volumes in each group were measured in situ within 4 weeks after injection. As expected, the tumor volumes were significantly increased in nude mice implanted with knocking down circPHACTR4 786-O cells compared with their corresponding control. While, knocking down of ERβ in 786-O cells significantly reduced tumor growth (Figure 7A). Then, after sacrificing the mice, the resected tumors were measured. The results revealed that targeting the circPHACTR4 with shcircPHACTR4 could partially reverse the ERβ-shRNA-suppressed ccRCC progression (Figure 7B–D). Importantly, our immunofluorescence staining demonstrated that c-Myc was decreased after knocking down of ERβ, and knocking down of circPHACTR4 could partially reverse the shERβ effect (Figure 7E).

Together, results from Figure 7A–E proved that ERβ may promote the ccRCC progression via altering the circPHACTR4 mediated miR-34b-5p/c-Myc signaling.

### 4 DISCUSSION

It has been found that ERβ could play important roles in the progression of ccRCC. Interestingly, results from these studies indicated that ERβ might function through different mechanisms. Our previous study identified that ERβ could promote ccRCC invasion via altering the circATP2B1/miR-204-3p/FN1 signals. Gu and colleagues demonstrated ERβ could promote ccRCC progression via altering the ANGPT-2/Tie-2 signaling-mediated angiogenesis. It has been reported that ERβ may play an important role in various types of tumors via CSCs. Due to their self-renewal capacities, CSCs are considered as the seeds of tumorigenesis, progression, and metastasis. It has been demonstrated that ERβ can promote tumor progression via CSCs in breast cancer and in papillary thyroid cancer. In addition, Zhang and colleagues reported that Nanog could play a role in mediating tobacco smoke-induced CSCs in ccRCC. Saeednejad Zanjani and colleagues reported that CD105 might promote ccRCC progression via CSCs. Accordingly, our study attempted to examine the role of ERβ-mediated CSCs in ccRCC through multiple approaches both in vitro and in vivo.

The circRNAs are known for their covalently closed loop structures without 5′ caps or 3′ poly A tails, which make them more stable. Therefore, the expression levels of circRNAs are much higher than their linear isomers. Today, we recognize that circRNAs are not the products of mis-splicing mRNAs, but play important roles in various diseases including cancers. Recently, a growing body of studies revealed that circRNAs may contribute to the occurrence and maintenance of cancers via CSCs. Gu and colleagues reported that circGprc5a could drive the self-renewal of bladder CSCs to promote...
bladder oncogenesis and metastasis.\textsuperscript{50} Qi and colleagues found that circ-ITCH sponged miR-214 could promote the self-renewal and stemness of CSCs by repressing the expression of Wnt-regulatory protein CTNNBP1 in lung adenocarcinomas.\textsuperscript{51} In addition, the communication between CSCs and their surrounding microenvironment is also quite important.\textsuperscript{52} Dai and colleagues found that exosomal circRNA\_100284 from arsenite-transformed human hepatic epithelial cells could be involved in the malignant transformation through circRNA\_100284/miR-217/EZH2 axis.\textsuperscript{53} However, the functional interactions between the circRNAs and miRNAs on the self-renewal and stemness of CSCs in ccRCC have not been fully elucidated.

In our study, we identified that ERβ could increase CSC population in ccRCC cells through binding to the promoter region of PHACTR4 and down-regulating circPHACTR4. There are many interaction mechanisms between circRNAs and other noncoding RNAs. One of the most classic functions of circRNAs is as a “miRNA sponge” in posttranscriptional regulation. The circRNA CRD1as contains 63 conserved miR-7 binding sites, which can inhibit the function of miR-7 and increase the expression levels of TGFBR2, SMAD, PARP, and other genes.\textsuperscript{54–56} Recently, Nan and colleagues reported a complex regulatory network between circRNAs, long noncoding RNAs (lncRNAs) and miRNAs.\textsuperscript{57} They found that IncRpa and circKar1 could directly and specifically bind to miR-671 to induce the up-regulation of caspase8 and p38 at the mRNA and protein levels. We previously reported that circRNAs could act as a miRNA “reservoir.”\textsuperscript{20} If more circRNA is expressed, more miRNA(s) will bind to the circRNA, thus increasing the availability of miRNA(s) for binding and inhibiting their target mRNAs. In our study, we found that circPHACTR4 could increase miR-34b-5p stability by acting as a miRNAs’ “reservoir” to partially reverse ERβ increased CSC population in ccRCC cells.

The miR-34b-5p has been reported to play important roles in a variety of tumors, such as breast cancer,\textsuperscript{58} pancreatic ductal adenocarcinoma,\textsuperscript{59} thyroid carcinoma,\textsuperscript{60} colitis-associated cancer,\textsuperscript{61} and diffuse large B-cell lymphoma.\textsuperscript{62} Importantly, it is reported that miR-34b-5p can serve as a biomarker to confirm the diagnosis of ccRCC.\textsuperscript{63} In our study, we found that circPHACTR4 may act as a reservoir of miR-34b-5p by stabilizing or promoting the transcription of miR-34b-5p to reduce the CSC population in ccRCC.

The proto-oncogene c-Myc is one of the most important transcription factors, and its abnormal expression and activation play pivotal roles in the initiation, maintenance, and progression of multiple cancers.\textsuperscript{43} It has been proposed that c-Myc regulates hundreds of different target genes, which are involved in numerous biological functions, such as cell proliferation, apoptosis, differentiation, tumor angiogenesis, and cell stemness.\textsuperscript{64,65} Importantly, c-Myc is one of the four Yamanaka factors used in reprogramming somatic cells to induce pluripotent stem cells from fibroblasts,\textsuperscript{66} which reveals that c-Myc may be a crucial regulator of CSCs.\textsuperscript{67} Recently, a growing body of studies show that c-Myc plays very important roles in the initiation and maintenance of CSCs in several kinds of tumors.\textsuperscript{68–70} Bioinformatics analysis of target genes predicts that c-Myc is one of the potential target genes of miR-34b-5p. In addition, Yang and colleagues reported that miR-34b-5p could target c-Myc in colitis-associated cancer.\textsuperscript{61} In this study, we confirmed miR-34b-5p could directly target the 3’ UTR of c-Myc to reduce CSC population in ccRCC cells.

In conclusion, we demonstrated that ERβ could promote CSC properties in ccRCC via altering the circPHACTR4/miR-34b-5p/c-Myc pathway, and blockage of this newly identified pathway substantially might reduce the acquisition of CSC features, providing potential therapeutic targets for ccRCC.

ACKNOWLEDGMENTS
We thank Chawnshang Chang at the University of Rochester Medical Center for editing the manuscript.

DISCLOSURES
The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS
Zhenwei Han, Zhan Yang, Shuyuan Yeh, and Yaxuan Wang were involved in conception, design, writing, review, and/or revision of the manuscript. Zhan Yang, Junfei Gu, Yanping Zhang, Xin Wang, and Zhihai Teng were involved in development of methodology. Yaxuan Wang, Zhan Yang, Dandan Wang, and Lei Gao were involved in analysis and interpretation of data. Dandan Wang, Lei Gao, and Yaxuan Wang were involved in administrative, technical, and material support. Wei Li and Shuyuan Yeh were involved in study supervision.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
The present study was authorized by the Ethics Committee of the Second Hospital of Hebei Medical University.

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