



PP2A drives the stemness in colorectal cancer cells by decreasing the Hippo signaling pathway

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ABSTRACT

Colorectal cancer (CRC) is one of the most prevalent malignancies. Accumulating evidence suggests that Hippo signaling pathway is a crucial signaling pathway that regulates stemness in multiple cancers, including CRC. Given that, the therapeutics targeting the Hippo pathway are of great interest. At the same time, previous studies have reported that PP2A regulates the Hippo pathway in tumorigenesis. In this study, we uncover the role of PP2A in vitro and in vivo and the interaction between PP2A and Hippo signaling pathway in CRC stemness. We investigate the molecular mechanism of PP2A-mediated Hippo pathway in CRC progression from the aspect of cell stemness. Furthermore, we explore the transcription factor of PP2A in CRC. In view of circRNA's regulation of cancer-related signaling pathways in CRC, we also study the association between PP2A-mediated Hippo pathway and the CRC-related circRNA (hsa-circ-001680) confirmed in previous studies. Of note, we demonstrate how hsa-circ-001680 regulates PP2A expression. The effect of hsa-circ-001680 on PP2A stability is investigated as well. In conclusion, the study demonstrates the mechanism of PP2A-mediated pathway on CRC stemness, which may provide new sights for the treatment of CRC.

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Introduction

CRC is the second leading cause of cancer-associated death, with an estimate of approximately 147,950 diagnoses with CRC and 53,200 deaths in the United States in 2020 (1). Various factors promote the risk of CRC, such as shortage of physical exercise, obesity, smoking habits, and so forth (2). Nowadays, treatment options for local and advanced disease have been increased, including endoscopic and surgery, extensive surgery for locoregional and metastatic disease, down-staging preoperative radiotherapy and systemic therapy, local ablative therapies for metastases, and palliative chemotherapy, targeted therapy, and immunotherapy (2). The hypothesis of cancer stem cells (CSCs) proposes that CSCs account for tumor initiation and progression. CSCs have been identified in several solid tumors, including CRC (3). Thus, it is of paramount significance to investigate the underlying mechanism of CRC progression associated with CSC for developing more effective therapeutic strategies.

The Hippo pathway is a vital pathway in charge of the proliferative and apoptosis of cancer cells (4). During early embryonic development, the Hippo signaling pathway regulates the growth and size of organs, and its functions lead to the coordinated balance of cell proliferation, apoptosis, and differentiation (5). Its main effectors, YAP (Yes-

associated protein) and TAZ (taffazin), regulate the signaling pathway through embryonic stem cells and determine cell fate and histogenesis (5). Dysfunction of Hippo pathway contributes to cancer development, including CRC (6,7). Moreover, there is accumulating evidence in support of Hippo pathway's regulating stemness in multiple cancers, such as breast cancer (8), CRC (9), etc. For instance, TFAP2C enhances CRC cell stemness and chemotherapeutic resistance via inactivating Hippo pathway. Given that, targeting Hippo pathway is a promising therapeutic strategy of CRC treatment.

As an important serine/threonine phosphatase, protein phosphatase 2A (PP2A) regulates many cellular phenotypes, such as cell proliferation, apoptosis, and signal transduction (10). Previous studies have reported that PP2A was involved in different cancers. For instance, PP2A was demonstrated to be a promising biomarker in endometrial cancer (11); stemness is accelerated by a SET/PP2A/E2F1 axis in gastric cancer (12); PP2A inhibits the migratory phenotype of cervical cancer cells by dephosphorylating p-JNK and p-p38 as well as the p-ERK/MAPK pathway (13). Also, PP2A was identified to regulate the Hippo pathway in tumorigenesis (14). However, the interaction of PP2A and Hippo pathway in CRC remains largely unclear.

Circular RNAs (circRNAs), a kind of endogenous non-coding RNAs (ncRNAs), are involved in the regulation

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of cancer development and have diagnostic and therapeutic potentials for various cancers, including CRC (15). Also, circRNA's regulation of cancer-related signaling pathways in CRC has been reported in previous studies. For example, hsa_circ_0128846 plays a promoting role in CRC via sponging hsa-miR-1184 and releasing AJUBA and curbing the activity of the Hippo/YAP pathway (16); cir-ITCH exerts an anti-tumor role in CRC via the regulation of Wnt/ β -catenin pathway (17). Therefore, this present study sought to reveal the association between the PP2A-mediated Hippo pathway and CRC-related circRNA in CRC cells.

In this study, we focused on the relationship between the Hippo pathway and the stemness of CRC cells via exploring the regulation mechanism of the PP2A/transcription factor of PP2A/Hippo/upstream circRNA axis in CRC.

Materials and Methods

Cell culture

CRC cell lines (HCT-116, DLD-1, SW480, CT-26, SW620, SW1116, HT29, and LOVO) as well as human colorectal epithelial cell line NCM460 were cultured in RPMI H1640 medium which contains 1% penicillin/streptomycin and 10% FBS. Both CRC cell lines and NCM460 cells were incubated in a humidified atmosphere with 5% CO₂ at the temperature of 37 °C.

qPCR

The extraction of RNA was performed by Trizol following the user guide of the manufacturer. M-MLV reverse transcriptase (Promega, Cat#: M1701)/TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher, Cat#: 4366596) was used to conduct reverse transcription of RNAs. qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Cat#: 4309155). Then, the 2^{- $\Delta\Delta$ Ct} method was employed to examine the relative expression levels. GAPDH and U6 were used as internal references. All the experiments were conducted in triplicate. The data were displayed as mean \pm standard deviation (SD).

Western blot

Cells were collected when cells grew to 80% confluence. Protein was extracted in RIPA buffer, followed by the isolation of SDS-PAGE. Then the prepared protein was transferred onto PVDF membranes and blocked by non-fat milk in TBST for 1 h at room temperature. Subsequently, the samples were incubated with primary antibodies overnight at 4 °C, followed by four washes in TBST (5 min per time). Then the samples were incubated with secondary antibodies on a slow shaker for 1 h. After washing, the membranes were subjected to electrochemiluminescence (ECL) liquid staining and photographed. GAPDH served as an internal reference.

Lentivirus production and cell transfection

The pLVX-IRES-Puro vectors for lentivirus-containing shRNAs directed against PP2A or hsa-circ-001680 were constructed for the follow-up experiments. 7 \times 10⁵ HEK293T cells were placed on the 6-well plates for 6h and transfected with pLKO.1-shRNA (1 μ g). After 48 h/72 h transfection, the supernatant solution was collected, fol-

lowed by centrifugation and filtration. The collected filtrate was a virus stock solution. The transfected cells in the log phase were seeded into the 96-well plates (2 \times 10⁴ cells/well) and incubated in fresh medium supplemented with the puromycin at the concentration of 0, 1, 2, 5, 10, or 15 μ g/mL overnight. The medium was replaced after overnight incubation and replaced again one week later, observing whether the cells were alive. The transfected cells were cultured in 24-well plates, and added with 105*MOI virus and 6 μ g/ml ploybrene (MOI > 10). 48 h later, the transfected cells were added to the selection medium and selected by 2 μ g/mL puromycin for 2 weeks. Then cell lines with stable PP2A or hsa-circ-001680 silence were constructed. qPCR was applied to verify the transfection efficiency.

Vector construction

The lentiviral vectors of shRNA directed against PP2A or hsa-circ-001680 and their corresponding negative control (sh-NC) were sub-cloned into pLKO.1 vector to construct interference vectors (sh-PP2A-1, sh-PP2A-2, sh-PP2A-3, and sh-NC; sh-hsa-circ-001680 and sh-NC). The sequence of PP2A was cloned into pcDNA3.1 vector to construct the overexpression vector pcDNA3.1/PP2A. The shNC and empty vector pcDNA3.1 were used as NC.

ALDH1 activity assay

The activity of ALDH1 (aldehyde dehydrogenase 1) was detected in the transfected SW480 and HT29 cells by use of the ALDH1 Activity Assay Kit under the guidance of the manufacturer's manual.

CCK8

The transfected cells (2 \times 10⁴ cells/mL) in the 96-well plates were incubated with 5% CO₂ at 37 °C for 24 h, 48h, and 72 h separately. CCK8 solution was added to each well. The value at the wavelength of 450 nm was detected with the application of a microplate reader.

Sphere formation

The transfected SW480 or HT29 cells were seeded into ultra-low attachment 6-well plates for two weeks with the treatment of DMEM/F12 medium without serum, supplemented with 2% B27, 5 μ g/mL insulin, 0.4% bovine serum albumin, 20 ng/mL basic fibroblast growth factor and 20 ng/mL epidermal growth factor. Image acquisition and counting of generated spheroids used a light microscope.

Xenograft nude model

All animal experiments in the study were conducted with the approval of the Institutional Ethics Committee of Chongqing Medical University. The nude mice obtained from Guangdong Medical Laboratory Animal Center were divided into two groups. 1 \times 10⁶ SW480 or HT29 cells transfected with sh-PP2A were subcutaneously injected into the flank of each experimental mouse while controls were injected with 1 \times 10⁶ SW480 or HT29 cells transfected with sh-NC per mouse. Tumor growth was monitored twice each week. Four weeks later, the mice were sacrificed. Then the tumors were collected for the measurement of tumor weight and volume as well as western blot.

Luciferase reporter assay

Wild-type PP2A promoter (PP2A promoter-WT) or mutant PP2A promoter (PP2A promoter-Mut) was sub-

jected to the sub-cloning into pGL3 luciferase reporter vector to construct pGL3-PP2A promoter vector. Then empty vector (PGL3) or pGL3-PP2A promoter vector was co-transfected with different plasmids including pcDNA3.1 or pcDNA3.1/hsa-circ-001680 or pcDNA3.1/hsa-circ-0001860 or pcDNA3.1/hsa-circ-0000677 or pcDNA3.1/circAGFG1 or pcDNA3.1/POLR2A into SW480 or HT29 cells.

DNA pulldown and mass spectrometry

The biotinylated PP2A promoter was mixed with cell lysates and placed on ice. The protein lysate was incubated with Streptavidin-agarose G beads for one hour at 4 °C, followed by cold FBS washing and centrifugation. Subsequently, beads were boiled with protein loading buffer at 100 °C for 10 min. The PP2A interacting proteins were treated with electrophoresis and silver staining. Finally, the PP2A-specific band was excised for mass spectrometry analysis.

ChIP

ChIP assay was performed using EZ-ChIP chromatin immunoprecipitation kit. Cells were fixed in 1% formaldehyde at room temperature for 10 min. Then cross-linking was quenched by adding glycine. Cross-linked cells were washed with cold PBS. Cell pellets were incubated in cell lysis buffer. Nuclei were re-suspended in 300 μ L Nuclear Lysis Buffer and sonicated. DNAs were subjected to decrosslinking, purification, and PCR analysis.

RNA pulldown

1 μ g biotin-labeled hsa-circ-001680 (Bio-hsa-circ-001680) was refolded in structure buffer, forming RNA secondary structure. RNA was denatured by heating at 95 °C for 2 min, cooled on ice for 3 min, and then left for 30 min at room temperature. 15 μ L streptavidin beads were added into the biotin-labeled hsa-circ-001680 for 2 hat 4 °C. Cell lysate (2×10^7 cells) was divided into three groups, Input, Bio-hsa-circ-001680, and Bio-NC. Beads-probe complexes were mixed with cell lysate and cultivated at 4 °C overnight, followed by centrifugation. Then, samples were subjected to 40 μ L RIP Wash Buffer, 10 μ L 5 \times SDS Loading Buffer at 95 °C, and shaking incubation (1000 rpm) for 10 min. Western blot was applied to analyze the eluted solutions.

RIP

After transfection, 6×10^7 cells were cultured in cell lysis buffer and then incubated with magnetic beads conjugated with antibodies against POLR2A (Anti-POLR2A) or antibodies against IgG (Anti-IgG). After 6-hour incubation at the temperature of 4 °C, remove the proteins of beads and detect the enrichment of purified RNA by qPCR after reverse transcription.

RNA stability analysis

After the transfection of sh-NC or sh-hsa-circ-001680, the SW480 cells were subjected to 50 mM α -amanitin to interfere with the synthesis of PP2A. Then, the qPCR analysis was applied to measure the level of PP2A after 5 h, 10 h, 15 h, 20 h, 25 h, 30 h. GAPDH served as a control.

Statistical analysis

All the experiments were performed in triplicate. Sta-

tistical analyses were conducted with SPSS 19.0 Software. Student's t-test was adopted for comparisons between two groups while one-way/two-way ANOVA followed by Turkey or Dunnett was utilized for comparisons among multiple groups. P value < 0.05 was considered statistically significant.

Results

PP2A is aberrantly elevated in CRC

To explore the role of PP2A in CRC, we first applied qPCR analysis to detect PP2A expression in NCM460 cells and CRC cell lines. The results showed the expression of PP2A is observantly up-regulated in CRC cell lines compared with normal colorectal cells (Figure 1), which demonstrated that PP2A may induce colorectal cancerogenesis.

PP2A enhances the stemness of CRC cells in vitro

Next, we investigated the effects of PP2A on CRC cell stemness. Firstly, we silenced and overexpressed PP2A. The knockdown as well as overexpression efficiencies of PP2A were verified by qPCR (Figure 2A). In view of the statistically significant knockdown efficiency of sh-PP2A-2/3 and the marked overexpression efficiency of pcDNA3.1-PP2A, we adopted the SW480 and HT29 cells transfected with sh-PP2A-2/3 or pcDNA3.1-PP2A for further study. To uncover the effect of PP2A on CRC cell stemness, we carried out a series of experiments. Considering the markedly high level of ALDH activity of CSCs, we measured the activity of ALDH1 (aldehyde dehydrogenase 1) in the indicated transfected SW480 and HT29 cells to access the stemness of CRC cells. Compared to the controls, ALDH1 activity was markedly decreased in sh-PP2A groups whereas significantly increased in pcDNA3.1-PP2A groups (Figure 2B). According to the results of CCK8, the OD values at 450 nm in the CRC cells transfected with sh-PP2A were lower than those of controls while pcDNA3.1-PP2A groups displayed higher ones (Figure 2C). In addition, sphere formation assays showed that the number of spheroids was largely decreased due to the down-regulation of PP2A while up-regulation of PP2A led to the opposite effect (Figure 2D). Moreover, in the PP2A-silenced and PP2A-overexpressed cells, we measured the levels of stemness-related proteins, including CD44 (Cluster of differentiation 44), SOX2 (SRY-box containing gene

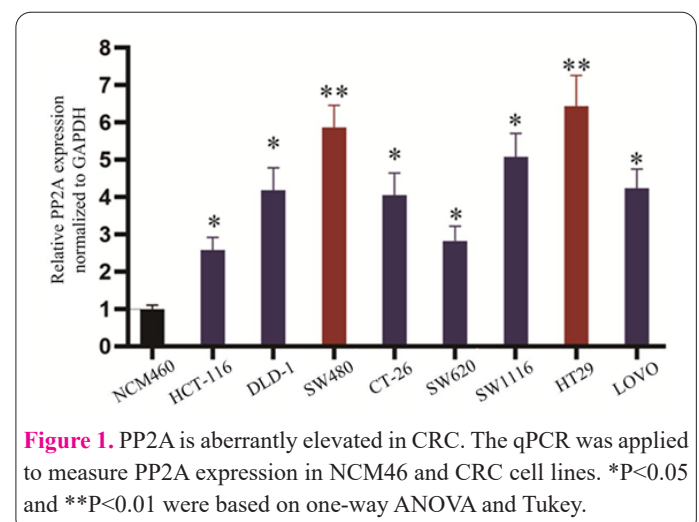


Figure 1. PP2A is aberrantly elevated in CRC. The qPCR was applied to measure PP2A expression in NCM46 and CRC cell lines. *P<0.05 and **P<0.01 were based on one-way ANOVA and Tukey.

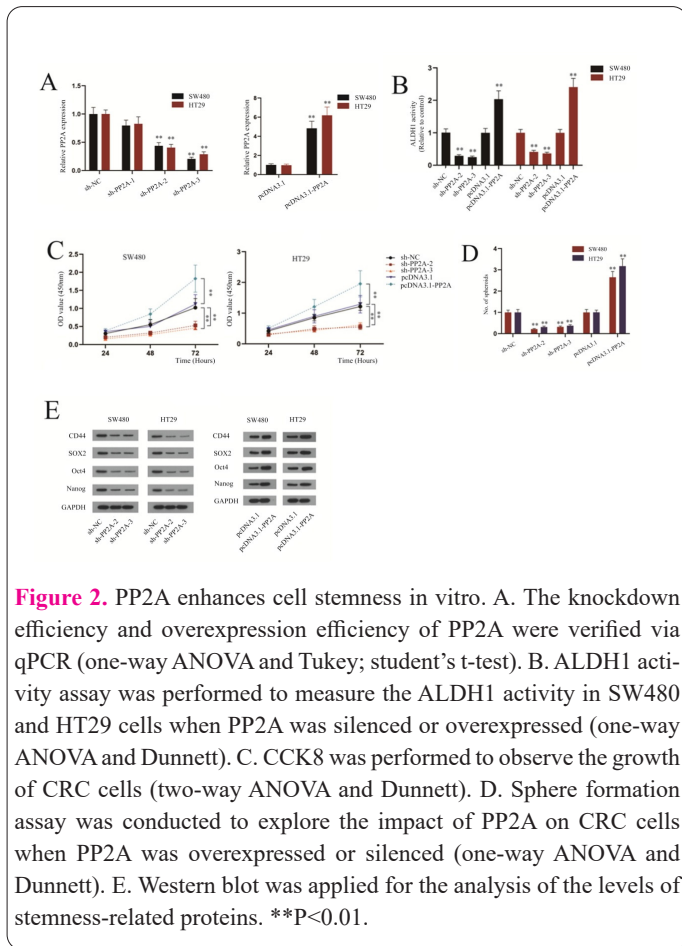


Figure 2. PP2A enhances cell stemness in vitro. A. The knockdown efficiency and overexpression efficiency of PP2A were verified via qPCR (one-way ANOVA and Tukey; student's t-test). B. ALDH1 activity assay was performed to measure the ALDH1 activity in SW480 and HT29 cells when PP2A was silenced or overexpressed (one-way ANOVA and Dunnett). C. CCK8 was performed to observe the growth of CRC cells (two-way ANOVA and Dunnett). D. Sphere formation assay was conducted to explore the impact of PP2A on CRC cells when PP2A was overexpressed or silenced (one-way ANOVA and Dunnett). E. Western blot was applied for the analysis of the levels of stemness-related proteins. **P<0.01.

2), Oct4 (Octamer-binding transcription factor 4), and Nanog (Nanog homeobox). The results showed that stemness-related protein levels were all obviously reduced in PP2A-silenced cells relative to controls while those were evidently increased in PP2A-overexpressed cells (Figure 2E). Taken together, low expression of PP2A inhibits the stemness of CRC cells while high expression of PP2A plays a promoting role in vitro.

Downregulation of PP2A plays an inhibitory role in the stemness of CRC cells in vivo

Furthermore, we constructed xenograft nude models to investigate the function of PP2A. Tumor weight and volume were both dramatically decreased when PP2A was silenced (Figure 3A, 3B). Also, the growth of tumor volume in the sh-PP2A group was relatively slower compared with sh-NC (Figure 3C). Plus, the Western blot analysis found that the stemness-associated protein levels of the tumor were all decreased when PP2A was down-regulated (Figure 3D). The above results elucidated that down-regulation of PP2A curbs the stemness of CRC cells in vivo.

PP2A promotes CRC tumorigenesis via inhibiting the Hippo pathway

With the help of the bioinformatics tool KEGG (<https://www.kegg.jp/>), it was found the involvement of PP2A in the Hippo pathway. Given that, we speculated that PP2A might enhance CRC cell stemness by suppressing the Hippo pathway. To confirm this assumption, we measured the level of Hippo pathway-related proteins in the transfected CRC cells (SW480 and HT29), including Mst1/2, YAP, and TAZ. In the Hippo pathway, Mst1/2 could up-regulate the expression of Last1/2 via phosphorylation,

and Last1/2 could phosphorylate YAP/TAZ, which would result in YAP/TAZ degradation, repressing tumorigenesis; PP2A could inhibit Mst1/2 and promote the expression of YAP/TAZ via dephosphorylation, propelling tumorigenesis. The data of Western blot revealed that when PP2A was silenced, the levels of Mst1/2, p-Last1/2, p-YAP, and p-TAZ were all improved while up-regulation of PP2A had the repressive effect (Figure 4A). Further, we measured the nuclear expressions of YAP and TAZ in SW480 and HT29 cells and found that the levels of YAP and TAZ in the nucleus were both reduced due to low expression of PP2A while those were increased because of ectopic expression of PP2A (Figure 4B). In sum, the above results demonstrated that PP2A promoted CRC tumorigenesis via suppressing the Hippo pathway.

Hsa-circ-001680 up-regulates PP2A expression via promoting its transcription to enhance CRC cell stemness

Previous studies have reported that several circRNAs contribute to cell stemness in CRC, such as hsa-circ-001680 (18), hsa-circ-0001806 (19), hsa-circ-0000677 (20), and circAGFG1 (21). Thus, we supposed that the abovementioned circRNAs may regulate cell stemness in CRC via modulating PP2A expression. To explore the association between circRNAs and PP2A, we knocked down candidate circRNAs and measured the levels of PP2A in

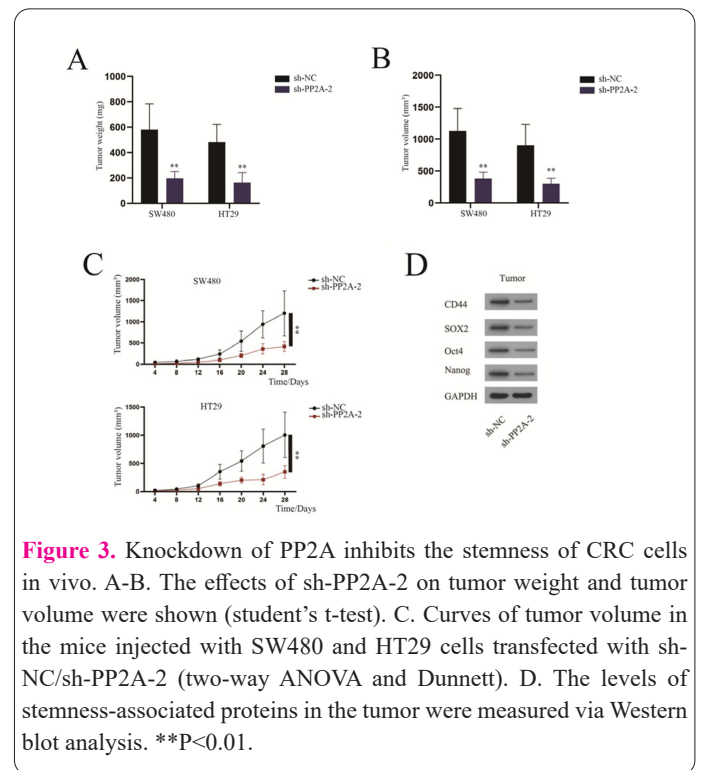


Figure 3. Knockdown of PP2A inhibits the stemness of CRC cells in vivo. A-B. The effects of sh-PP2A-2 on tumor weight and tumor volume were shown (student's t-test). C. Curves of tumor volume in the mice injected with SW480 and HT29 cells transfected with sh-NC/sh-PP2A-2 (two-way ANOVA and Dunnett). D. The levels of stemness-associated proteins in the tumor were measured via Western blot analysis. **P<0.01.

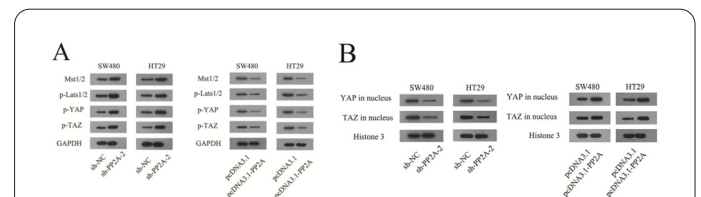


Figure 4. PP2A promotes CRC tumorigenesis via inhibiting the Hippo pathway. A. Western blot analysis of Hippo pathway-related protein levels when PP2A was inhibited or overexpressed. B. The effects of PP2A on the protein levels of YAP/TAZ in the nucleus were detected via Western blot. Histone 3 served as an endogenous control.

SW480 and HT29. The results showed that the ablation of hsa-circ-001680 observantly reduced the levels of PP2A (Figure 5A). Plus, Western blot analysis also showed hsa-circ-001680 knockdown negatively mediates PP2A expression (Figure 5B). Furthermore, to determine the regulatory role of hsa-circ-001680 on PP2A, we overexpressed circRNAs and carried out dual luciferase reporter assays in SW480 and HT29 cells. The results showed that pcDNA3.1-hsa-circ-001680 largely promoted luciferase activity, suggesting that hsa-circ-001680 could promote the transcription activity of the PP2A promoter (Figure 5C). From the above, hsa-circ-001680 positively mediates PP2A expression via propelling its transcription.

POLR2A is transcription factor of PP2A

To further investigate the transcription factor of PP2A, we carried out a biotinylated DNA pulldown assay followed by silver staining in SW480 and obtained the specific band, which was subjected to mass spectrometry analysis. The result showed that POLR2A might be the transcription factor of PP2A (Figure 6A). UCSC (<http://genome.ucsc.edu/>) also predicted that POLR2A is one of the transcription factors of PP2A. To explore the effect of POLR2A on PP2A, we found that luciferase activity was largely enhanced in the cells co-transfected with pcDNA3.1-POLR2A and pGL3-PP2A promoter vector

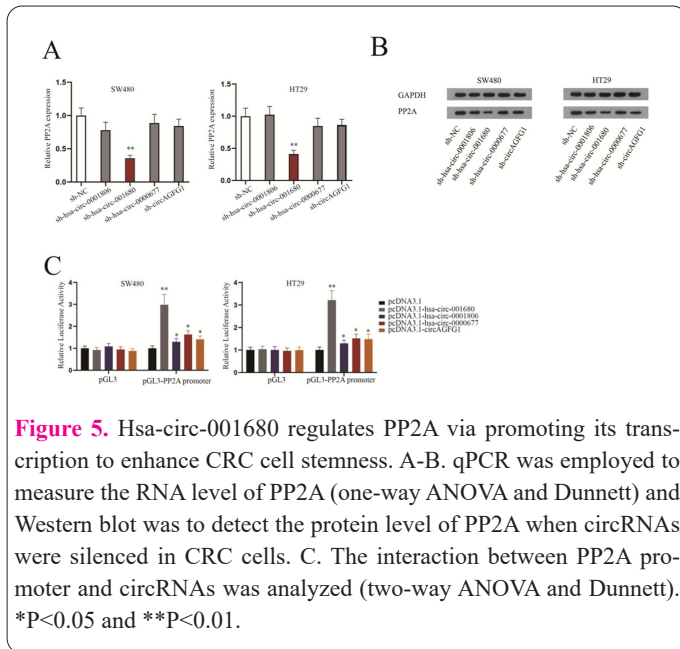


Figure 5. Hsa-circ-001680 regulates PP2A via promoting its transcription to enhance CRC cell stemness. A-B. qPCR was employed to measure the RNA level of PP2A (one-way ANOVA and Dunnett) and Western blot was to detect the protein level of PP2A when circRNAs were silenced in CRC cells. C. The interaction between PP2A promoter and circRNAs was analyzed (two-way ANOVA and Dunnett). * $P < 0.05$ and ** $P < 0.01$.

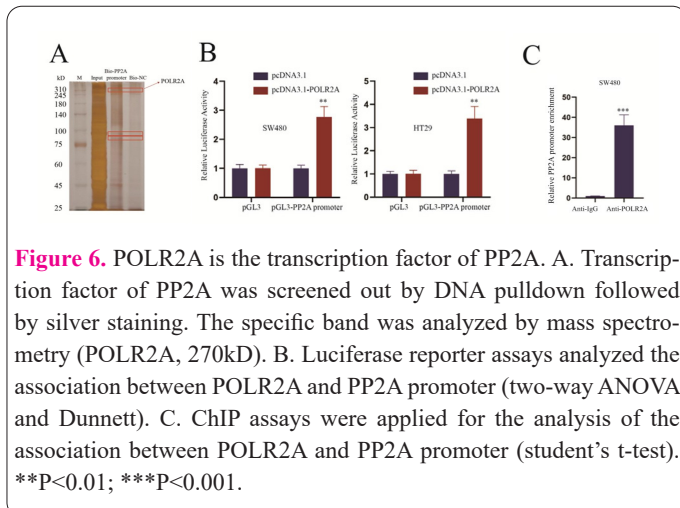


Figure 6. POLR2A is the transcription factor of PP2A. A. Transcription factor of PP2A was screened out by DNA pulldown followed by silver staining. The specific band was analyzed by mass spectrometry (POLR2A, 270kD). B. Luciferase reporter assays analyzed the association between POLR2A and PP2A promoter (two-way ANOVA and Dunnett). C. ChIP assays were applied for the analysis of the association between POLR2A and PP2A promoter (student's t-test). ** $P < 0.01$; *** $P < 0.001$.

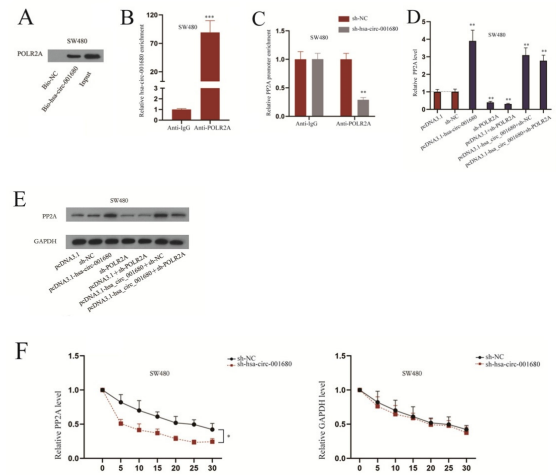


Figure 7. Hsa-circ-001680 up-regulates PP2A expression via recruiting POLR2A and post-transcriptional regulation, and promotes the stability of PP2A. A. RNA pulldown assay was applied for the detection of the expression of POLR2A via biotinylating hsa-circ-001680. B. RIP assay was conducted to verify the regulation role of hsa-circ-001680 on POLR2A (student's t-test). C. ChIP assay detected the enrichment of PP2A promoter in anti-POLR2A complexes when hsa-circ-001680 was silenced (one-way ANOVA and Dunnett). D-E. The level of PP2A in SW480 cells transfected with pcDNA3.1, sh-NC, pcDNA3.1-hsa-circ-001680, sh-POLR2A, pcDNA3.1+sh-POLR2A, pcDNA3.1-hsa-circ-001680+sh-NC, or pcDNA3.1-hsa-circ-001680+sh-POLR2A was measured via qPCR (one-way ANOVA and Turkey) and Western blot. F. qPCR was applied to detect PP2A level in the SW480 cells transfected with sh-NC or sh-hsa-circ-001680 (two-way ANOVA and Dunnett). * $P < 0.05$ and ** $P < 0.01$.

compared with controls (Figure 6B), suggesting POLR2A could up-regulate the expression of PP2A. Also, the ChIP assay further verified this finding as the PP2A promoter was preferentially enriched in the Anti-POLR2A group (Figure 6C). Altogether, the above results demonstrated that POLR2A is the transcription factor of PP2A.

Hsa-circ-001680 up-regulates PP2A expression via recruiting POLR2A and post-transcriptional regulation and promotes the stability of PP2A

To determine whether hsa-circ-001680 could bind with POLR2A, we performed RNA pulldown and RIP assays in SW480, followed by Western blot and qPCR analyses respectively. The data showed that POLR2A was clearly enriched in the biotin-labeled hsa-circ-001680 group relative to the Bio-NC group (Figure 7A). Meanwhile, hsa-circ-001680 was significantly abundant in the Anti-POLR2A group (Figure 7B). Besides, we measured the enrichment of PP2A promoter in the ChIP assay after transfection with sh-NC or sh-hsa-circ-001680, and POLR2A antibody or IgG antibody. The results showed that relative PP2A promoter enrichment in Anti-POLR2A bound complexes was obviously decreased after silencing hsa-circ-001680 (Figure 7C). Additionally, to further determine whether hsa-circ-001680 regulates the expression of PP2A via binding with POLR2A or directly mediates it from the post-transcriptional level, we performed rescue experiments and analyzed the levels of PP2A in the SW480 cells transfected with the indicated plasmids. The data of qPCR analysis showed POLR2A knockdown did not thoroughly reverse the up-regulation effect of hsa-

circ-001680 on PP2A expression (Figure 7D), which suggested that hsa-circ-001680 positively regulates POLR2A from the post-transcriptional level. The results of Western blot analysis verified this finding as well (Figure 7E).

Further, we investigated whether hsa-circ-001680 affects the stability of PP2A and found that the down-regulated hsa-circ-001680 reduced the half-life of PP2A level from 18 h to 5 h (Figure 7F), which indicated that hsa-circ-001680 conduces to stabilizing PP2A.

To sum up, hsa-circ-001680 recruits POLR2A to up-regulate PP2A. In the meantime, hsa-circ-001680 directly up-regulates PP2A expression from the post-transcriptional level. Besides, hsa-circ-001680 contributes to the stability of PP2A.

Discussion

CRC is one of the most prevalent malignancies, featuring a progressive accumulation of genetic and epigenetic abnormalities (22). Although great advancements in treatment options have been achieved, the overall survival of advanced CRC patients remains low (23). So it's of great importance to explore the molecular mechanism underlying CRC development and progression. Multiple lines of evidence have demonstrated that CSCs drive tumor initiation; CSCs have been the focus of research on various cancers, including CRC (3). Therefore, we investigated the molecular mechanism underlying CRC cell stemness.

In addition, amounts of studies reckon targeting the Hippo tumor-suppressor pathway as a promising treatment strategy, including CRC. For instance, TFAP2C accelerates CRC stemness and chemotherapeutic resistance through inactivating the Hippo signaling pathway (9); RASAL2 facilitates CRC development via the LATS2/YAP1 axis of Hippo signaling pathway (24); ZNF280A enhances cell proliferative phenotype and tumorigenicity via curbing Hippo-signaling pathway in CRC (25). Thus, it is worthwhile to investigate the effect of the Hippo pathway on CRC cell stemness. At the same time, the involvement of PP2A in the regulation of the Hippo pathway in tumorigenesis has been confirmed in previous researches (14). So we probed into the effects of the PP2A-mediated Hippo pathway on CRC cell stemness.

Furthermore, increasing studies have reported that circRNAs contribute to cancer development. For example, circ_0026344 functions as a prognostic biomarker and represses the progression of CRC through miRNA-21 and miRNA-31 (26); circ_001569 promotes the proliferation and invasion of CRC (27); hsa_circRNA_103809 regulates CRC cell proliferative and migratory phenotypes via miR-532-3p/FOXO4 axis (28,29). Herein, we explored the relationship between the PP2A-mediated Hippo pathway and CRC-related circRNAs.

Our study found that PP2A is aberrantly abundant in CRC cells via qPCR analysis. To investigate the effects of PP2A on CRC cell stemness, we performed ALDH1 activity detection, sphere formation, and western blot analysis of stemness-related genes as well as in vivo assays. The results demonstrated that PP2A enhances the stemness of CRC cells. Also, we found the involvement of PP2A in the Hippo pathway in CRC progression with the help of KEGG and confirmed that PP2A promotes CRC tumorigenesis via inhibiting the Hippo pathway by western blot analysis of Hippo-related proteins. Plus, qPCR and wes-

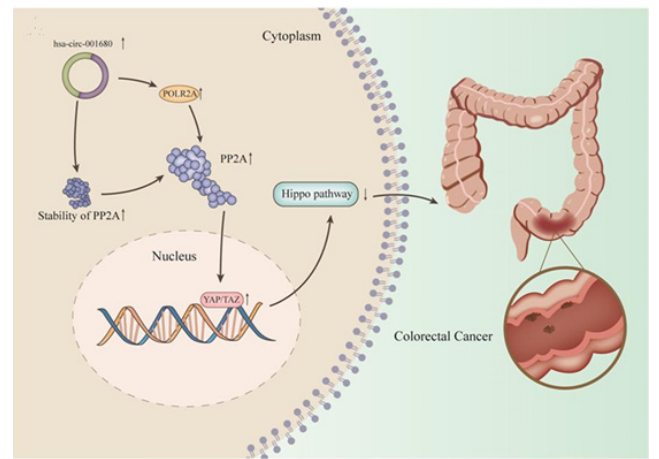


Figure 8. Hsa-circ-001680 up-regulates PP2A expression via recruiting POLR2A and post-transcriptional regulation and promotes the stability of PP2A. The schema for the mechanism of hsa-circ-001680/PP2A/POLR2A/Hippo axis in CRC.

tern blot analyses were employed to preliminarily screen out upstream targets of PP2A after knocking down candidate circRNAs. Subsequently, luciferase reporter assay confirmed that hsa-circ-001680 is the upstream target of PP2A as hsa-circ-001680 promoted the luciferase activity of the pGL3-PP2A promoter. POLR2A as the transcription factor of PP2A was verified and confirmed through DNA pulldown, luciferase reporter and CHIP assays. Additionally, RNA pulldown and RIP assays verified the interaction between hsa-circ-001680 and POLR2A while the CHIP assay confirmed the interaction between PP2A, POLR2A and hsa-circ-001680. To further probe into the interaction of hsa-circ-001680/POLR2A/PP2A axis, we performed rescue experiments. We found that hsa-circ-001680 could recruit POLR2A to up-regulate PP2A. Meantime, hsa-circ-001680 could directly up-regulate PP2A expression from the post-transcriptional level. Also, hsa-circ-001680 could stabilize PP2A. From the above, hsa-circ-001680 recruits the transcription factor POLR2A to the promoter of PP2A to induce a high expression of PP2A or positively modulates PP2A expression from the post-transcriptional level which results in YAP/TAZ's entering the nucleus, further inhibiting Hippo pathway, and finally inducing CRC (Figure 8). In conclusion, our present study provided new sights and promising targets for CRC therapy.

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