

# Baicalein Suppressed Cervical Cancer Tumorigenesis by Modulating circ\_0007364/microRNA-665/Tumor Protein D52 Axis

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## Liu *et al.*: Baicalein Suppressed Cervical Cancer Tumorigenesis

Baicalein has anti-tumor effect and participates in multi-channel regulation mechanism. However, the mechanism of baicalein action has not been fully clarified in cervical cancer. Tumor protein D52, circ\_0007364 and microRNA-665 expression were determined by quantitative reverse transcription-polymerase chain reaction. Tumor protein D52 and apoptosis-related proteins were detected by Western blot. Cell proliferation was measured *via* methyl thiazolyl tetrazolium, 5-ethynyl-2'-deoxyuridine and colony formation assays. The associations among microRNA-665, circ\_0007364 and tumor protein D52 were determined by dual luciferase report assay. Cell invasion and apoptosis were assessed by Transwell assay and flow cytometry. The function experiments were carried out *in vivo* and *in vitro*. Baicalein significantly decreased circ\_0007364 and tumor protein D52 expression and increased microRNA-665 expression in cervical cancer cell samples. Circ\_0007364 or tumor protein D52 introduction and microRNA-665 down regulation weakened the suppressive effects of baicalein in cervical cancer cells. Moreover, circ\_0007364 functioned by binding to microRNA-665 to regulate tumor protein D52 and affect cellular process in cervical cancer. Our study showed that baicalein inhibited cervical cancer cell growth *via* regulating circ\_0007364/microRNA-665/tumor protein D52 axis, further understanding the anticancer mechanism of baicalein and contributing to find more effective anticancer drug components for cervical cancer.

**Key words:** Baicalein, circ\_0007364, microRNA-665, tumor protein D52, cervical cancer

Cervical Cancer (CC) mainly occurs in the cervix and poses a serious threat to women's life, constituting a leading cause of morbidity<sup>[1,2]</sup>. Moreover, most CCs are caused by Human Papillomavirus (HPV)<sup>[3]</sup>. The treatments of CC are mainly surgery and chemoradiotherapy<sup>[2]</sup>. Thus, exploring the treatment methods of CC and understanding the molecular mechanism will contribute to develop new strategies to hind CC.

Baicalein is a bioactive flavonoid used as the main active component of traditional Chinese medicine and its application effect in tumor treatment is very significant<sup>[4]</sup>. Various studies have proved that baicalein has anti-tumor effect and participates in multi-channel regulation mechanism in cancers, including Hepatocellular Carcinoma (HCC), breast cancer and CC<sup>[5-7]</sup>. For example, Wu *et al.*<sup>[8]</sup> pointed

out that baicalein suppressed mammalian Target of Rapamycin Complex 1 (mTORC1) inhibitor-induced autophagy and could selectively promote the chemical sensitivity of Tumor-Initiating Cells (TICs) and HCC without affecting mouse and human primary hepatocytes. In addition, baicalein regulated breast cancer apoptosis and autophagy, so as to achieve anticancer effect<sup>[6]</sup>. Moreover, baicalein affected circ Hippocampus Abundant Transcript 1 (circHIAT1)/microRNA (miR)-19a-3p pathway to inhibit cell growth in CC<sup>[9]</sup>. Nevertheless, how baicalein acts in CC remains to be explored. Understanding the

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anticancer molecular mechanism of baicalein will help to improve the treatment efficiency and treatment methods in CC.

Circular Ribonucleic Acid (circRNA) is a noncoding RNA that plays an indispensable role in cancers<sup>[10-12]</sup>. Interestingly, circRNA does not play a role alone in cells, but combines with miRNA to affect messenger RNA (mRNA) transcription, thus affecting the development and progress of cells<sup>[13,14]</sup>. With the deepening of research, circRNA was found to regulate cell development and disease development, including breast cancer, HCC, colorectal cancer, immune diseases and CC<sup>[15-19]</sup>. For example, circ\_100367 bound to miR-217 to regulate Wnt3, further modulating esophageal squamous cell carcinomas cell growth<sup>[20]</sup>. Circ\_101996 promoted CC cell proliferation by inducing miR-8075-dependent Targeting Protein for Xenopus Kinesin-like Protein 2 (TPX2) expression<sup>[21]</sup>. Previous work showed a high circ\_0007364 expression in CC<sup>[22]</sup>. Interestingly, in our study, baicalein significantly decreased circ\_0007364 expression, but the related regulatory pathway in CC has not been fully clarified. In this paper, we found that circ\_0007364 content was reduced in baicalein-treated CC cells. Through further prediction and verification, we determine that baicalein inhibited cell growth and invasion through circ\_0007364/miR-665/Tumor Protein D52 (TPD52) axis in CC.

## MATERIALS AND METHODS

### Samples collection:

Twenty seven pairs of CC tissues and matched normal cervical tissue were collected from patients who have undergone a surgical resection in Wuchang Hospital Affiliated to Wuhan University of Science and Technology. The experiment has been approved by the ethics committee of Wuchang Hospital Affiliated to Wuhan University of Science and Technology, and written informed consent has been obtained from all patients. Collected tissues were stored in a refrigerator.

### Cell culture, treatment and transfection:

SiHa, HeLa and Ect1/E6E7 cells were purchased from EK-Bioscience (Shanghai, China) and cultured in Dulbecco's Modified Eagle's medium (DMEM) contained 10 % Fetal Bovine Serum (FBS) and streptomycin/penicillin with 5 % Carbon dioxide (CO<sub>2</sub>) at 37° for followed experiments.

Circ\_0007364 mimic (circ\_0007364), small interfering RNA (siRNA) against circ\_0007364 (si-circ\_0007364), plasmid cloning Deoxyribonucleic Acid (pcDNA)-TPD52 and negative controls were obtained from GenePharma (Shanghai, China). MiR-665 mimic, miR-665 inhibitor and negative controls were purchased from Ribo Bio (Guangzhou, China). When the confluence of cells reached 70 %~90 %, cells were transfected into CC cells *via* Lipofectamine 2000 (Invitrogen, Carlsbad, California, United States of America (USA)). SiHa and HeLa cells were treated with baicalein (0, 10, 20, and 30 µg/ml) for 48 h and then were collected for the next experiments. Finally, 20 µg/ml baicalein was used to function experiments.

### Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR):

Collected cell and tissue RNA were extracted with TRIzol (Invitrogen). After that, RNA quality was assessed through the NanoDrop™ 2000c. According to the detection concentration, the volume required for RNA was calculated and 1 µg RNA was reversely transcribed into complementary Deoxyribonucleic Acid (cDNA) utilizing high-capacity reverse transcription reagents (Qiagen, Hilden, Germany). Then, the circRNA and mRNA expression was detected SYBR Premix Ex Taq™ II (Invitrogen). TaqMan miRNA assay was applied to detect miR-655. U6 was used as the control for miR-665. Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) was used as the control for circ\_0007364, Protein Tyrosine Phosphatase 4A2 (PTP4A2) and TPD52. The primers are shown in Table 1. Gene expression was measured through calculating with 2<sup>-ΔΔCt</sup> method.

**TABLE 1: PRIMER SEQUENCES USED FOR QRT-PCR**

Name	Primers for PCR (5'-3')
hsa_circ_0007364	F-GTGACGACTTTGGTTCGAGT R-CTCACTTGTGACGCGAAATGC
TPD52	F-GCAAGACGTGACAGCAACAT R-TTGCAGGTCCCATCTGTGTAAG
miR-665	F-GCGTATGAACCAGGAGGCTGAG R-CTCAACTGGTGTCGTGGAG
GAPDH	F-GACAGTCAGCCGCATCTTCT R-GCGCCCAATACGACCAAATC
U6	F-CTCGCTTCGGCAGCACA R-AACGCTTCACGAATTTGCGT
PTP4A2	F-GGAGTGACGACTTTGGTTTCG R-TCAAAGCAAGTGCAACCAGC

**RNase R treatment:**

After RNA extraction, added 3 U/ $\mu$ g Ribonuclease R (RNase R) (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 37° for 15 min. The control sample of RNA was incubated without RNase R.

**Western blot assay:**

Total protein extraction was performed using Radioimmunoprecipitation Assay (RIPA) lysis buffer and then Bicinchoninic Acid (BCA) kits (Thermo Fisher, Carlsbad, California, USA) were utilized for protein concentration analysis. Subsequently, Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed to separate protein on the midi-cell electrophoresis system and then the protein was transferred to nitrocellulose membrane. The membranes were incubated with anti-GAPDH (1:1000 dilution; Cat. No: AF0006; Beyotime, Shanghai, China), anti-cleaved-caspase-3 (1:1000; Cat. No: AB2302, Abcam, Cambridge, Massachusetts, USA), anti-Matrix Metalloprotease-9 (MMP-9) (1:1000; cat. no. ab219372, Abcam) and anti-TPD52 (1:1000 dilution; Cat. No: AB182578, Abcam) at 4° overnight. Then the membrane was incubated with anti-rabbit or anti-mouse Immunoglobulin G (IgG) (1:1000 dilution; Cat. No: AB228414, Thermo Fisher). Finally, proteins blot was detected using an Electrochemiluminescence (ECL) system (Thermo Fisher).

**Colony formation assay:**

Transfected cells were seeded into six-well petri dishes added with DMEM and then went through 2 w incubation. Then cells were fixed with 4 % paraformaldehyde (Phygene, Fuzhou, China), followed by the staining with crystal violet (Sigma). Finally, cell colonies were assessed under microscope.

**Methyl Thiazolyl Tetrazolium (MTT) assay:**

Transfected cells were seeded into 96-well petri dishes added with DMEM and then went through 72 h incubation at 37°. Then, each well was added with 20  $\mu$ l MTT solution (Sigma-Aldrich) and incubated for 4 h. After each well was added with 200  $\mu$ l dimethyl sulfoxide (Sigma), each sample was measured at 490 nm.

**Transwell invasion assay:**

Transfected cells grew in the upper chamber of Transwell (six-well plate, Corning Costar, Lowell, Massachusetts, USA), and the Transwell inserts were coated with Matrigel. Complete DMEM medium was added into the bottom chamber. After incubation for 48 h, the invasion cells were fixed and stained with 0.1 % crystal violet (Sigma-Aldrich) solution and then counted on microscope.

**Hematoxylin and Eosin (HE) staining:**

The tissue was fixed with 4 % formalin and dehydrated with gradient concentration ethanol. Subsequently, the tissue was paraffin-embedded and the tissue section was cut into 4  $\mu$ m sections. Subsequently, the sections were stained with 3,3-Diaminobenzidine (DAB) and then subjected to counterstaining with hematoxylin. Finally, a microscope was used for observation.

**5-Ethynyl-2'-Deoxyuridine (EdU) incorporation assay:**

Transfected CC cells were incubated with 20  $\mu$ M EdU reagent (RiboBio, Guangzhou, China) in 96-well plates for 2 h. Subsequently, cells were fixed with 4 % paraformaldehyde (Phygene) and stained in 5  $\mu$ g/ml 4,6-Diamidino-2-Phenylindole (DAPI). After incubation for 30 min in the dark, EdU-positive cells were observed by fluorescence microscope.

**Dual luciferase report assay:**

Circ\_0007364 and TPD52 3' Untranslated Regions (3' UTRs) contained binding sites of miR-665 were synthesized and inserted into the psiCHECK-2 vector (Promega, Madison, Wisconsin, USA), named WT-circ\_0007364 or WT-TPD52 3'UTR. In addition, the circ\_0007364 or TPD52 3'UTR contained miR-665 mutated sites were synthesized and then were inserted into the psiCHECK-2 vector (Promega), named Mutant-Type (MUT)-circ\_0007364 or MUT-TPD52 3'UTR. These vectors were co-transfected with miR-NC or miR-665 mimic (miR-665) into cells *via* Lipofectamine 2000 (Invitrogen). At 48 h after culture, the firefly and Renilla luciferase activities acted as internal control.

**Flow cytometry analysis:**

Cell apoptosis was assessed by the FITC apoptosis

detection kit (BD Biosciences, Franklin Lakes, New Jersey, USA).  $2 \times 10^5$  transfected CC cells were seeded into well and incubated with 5  $\mu$ l Annexin V-Fluorescein Isothiocyanate (FITC) and 5  $\mu$ l Propidium Iodide (PI) in the dark. Eventually, cell apoptosis was measured using FACSscan® flow cytometry (BD Biosciences).

### Xenografts experiments:

This animal experiment was approved by the Ethics Committee of Wuchang Hospital Affiliated to Wuhan University of Science and Technology. HeLa cells ( $2 \times 10^5$ ) transfected with sh-circ\_0007364 or sh-NC were subcutaneously injected into the right back of Bagg and Albino (BALB/c) female nude mice (4 w-5 w, n=24). The experiment was divided into four groups. After 8 d, baicalein (45 mg/kg) was given every 3 d and tumor volume was observed and measured every 3 d. 23 d later, the tumor was taken out, the tumor growth curve and tumor weight were obtained.

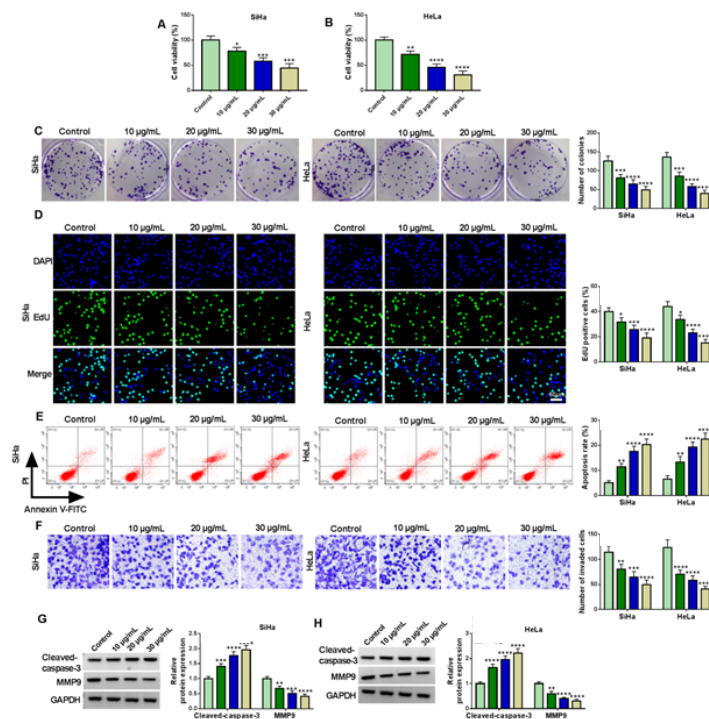
### Statistical analysis:

Statistical analysis was displayed *via* using GraphPad Prism 7.0 software (GraphPad, Bethesda, Maryland, USA). Pearson correlation analysis,

Student's t-test or one-way analysis of variance was used for comparison. Statistical significance was indicated when  $p < 0.05$ .

## RESULTS AND DISCUSSION

To understand whether baicalein showed inhibitory effects on CC cells, we selected two CC cell lines routinely used in CC research and treated them with different concentrations of baicalein (10, 20, 30  $\mu$ g/ml). Then, the physiological indexes of cells were measured. The results of fig. 1A-fig. 1D showed that the cell viability and cell colony number and EdU positive cells were obviously decreased with the increase of baicalein concentration, indicating that baicalein inhibited CC cell proliferation. Subsequently, CC cell apoptosis was gradually increased with increasing baicalein concentration (fig. 1E). Results of Transwell showed that the higher the concentration of baicalein, the greater the inhibitory effect on CC cell invasion (fig. 1F). Baicalein significantly induced cleaved-caspase-3 and obviously inhibited MMP-9 production in CC cells (fig. 1G and fig. 1H). In conclusion, baicalein inhibited the growth of CC cells at different concentrations, indicating that it could be applied for the treatment of CC.



**Fig. 1:** Baicalein inhibited CC cell proliferation, (A to D): Cell proliferation was detected under different concentration of baicalein (control, 10, 20, 30  $\mu$ g/ml) in CC cells; (E): Cell apoptosis was measured under different concentration of baicalein in CC cells using flow cytometry; (F): Cell invasion were measured under different concentration of baicalein in CC cells using Transwell assay; (G) Cleaved-caspase-3 and (H): MMP expression were measured under different concentration of baicalein in CC cells using Western blot assay

Note: (■): Control; (■): 10  $\mu$ g/ml; (■): 20  $\mu$ g/ml and (■): 30  $\mu$ g/ml



To determine circ\_0007364 expression property in CC, we collected 27 pairs of CC tissues and their adjacent tissues. The staining in tumor tissues was deepened in fig. 2A. Circ\_0007364 content was elevated in CC tissues and cells (fig. 2B and fig. 2C). As shown in fig. 2D and fig. 2E, after RNase R treatment, circ\_0007364 was more stable than its linear mRNA (PTP4A2). Taken together, circ\_0007364 was highly expressed in CC.

Combined with the above research, we speculated that circ\_0007364 was related to baicalein in CC cells growth. Therefore, we chose baicalein to treat CC cells and observe whether circ\_0007364 expression was changed. As shown in fig. 3A, with the increase of baicalein treatment concentration, circ\_0007364 content was gradually reduced. Therefore, we speculated that baicalein decreased circ\_0007364 expression to inhibit the growth of CC. To prove this idea, we built the overexpression

cell line of circ\_0007364 (circ\_0007364) and control cell line (pCD5-ciR). The expression of circ\_0007364 increased significantly (fig. 3B). Next, 20  $\mu\text{g/ml}$  baicalein was used to treat transfected or non-transfected CC cells and baicalein inhibited circ\_0007364 expression, but circ\_0007364 transfection attenuated the inhibitory effect (fig. 3C). Consistent with previous studies<sup>[22]</sup>, baicalein inhibited the proliferation of CC cells (fig. 3D-fig. 3F), promoted apoptosis (fig. 3G), weakened the ability of cell invasion and metastasis (fig. 3H), enhanced cleaved-caspase-3 production and reduced MMP9 protein expression (fig. 3I and fig. 3J). However, enhanced circ\_0007364 expression reverses the suppression effects of baicalein on CC cell progression (fig. 3C to fig. 3J). Therefore, promotion of circ\_0007364 significantly attenuated the inhibitory influence of baicalein in CC cell lines.

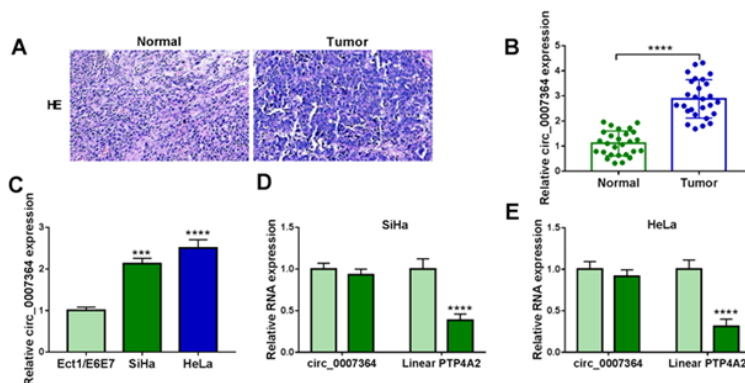


Fig. 2: Circ\_0007364 content in CC, (A): Circ\_0007364 expression in tumor and normal tissues; (B and C): Circ\_0007364 content was detected using qRT-PCR and (D and E): qRT-PCR analysis for circ\_0007664 and PTP4A2 expression after RNase R in CG cells  
Note: (■): Mock and (■): RNase R

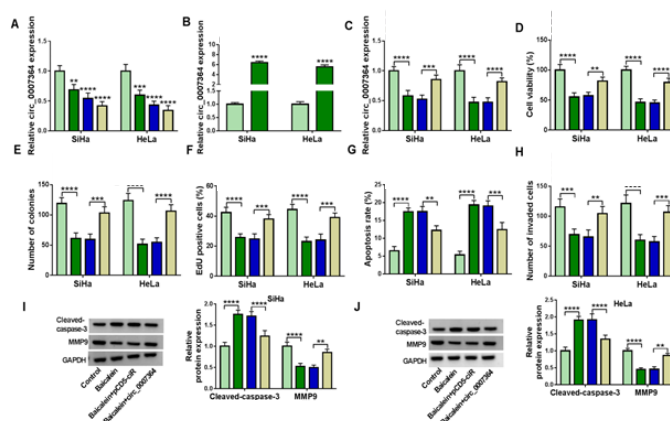
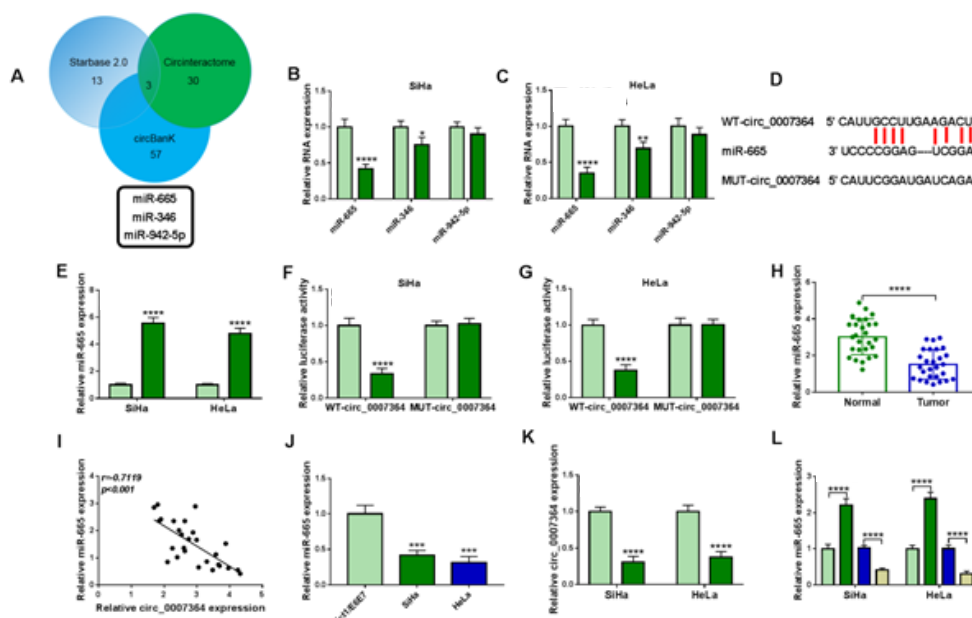


Fig. 3: Circ\_0007364 suppressed baicalein-mediated CC cell apoptosis, (A): Circ\_0007364 was detected; (B): Circ\_0007364 transfection induced circ\_0007364 expression; (C): Circ\_0007364 content was detected in control, baicalein, baicalein+pCD5-ciR and Baicalein+circ\_0007364 groups of CC cells; (D to F): Cell proliferation was detected in control, Baicalein, Baicalein+pCD5-ciR and Baicalein+circ\_0007364 groups of CC cells; (G): Cell apoptosis was measured in control, Baicalein, Baicalein+pCD5-ciR and Baicalein+circ\_0007364 groups of CC cells using flow cytometry; (H): Cell invasion were measured using transwell assay and (I and J): Cleaved-caspase-3 and MMP expression were assessed in control, Baicalein, Baicalein+pCD5-ciR and Baicalein+circ\_0007364 groups of CC cells using Western blot assay  
Note: (A): (■): Control; (■): 10  $\mu\text{g/ml}$ ; (■): 20  $\mu\text{g/ml}$  and (■): 30  $\mu\text{g/ml}$ ; (B): (■): pCD5-ciR and (■): circ-0007364 and (C): (■): Control; (■): Baicalein; (■): Baicalein+pCD5-ciR and (■): baicalein+circ-0007364

After the prediction of Star base 2.0, circBank and Circinteractome, we found three candidate miRNAs of circ\_0007364, including miR-665/-346/-942-5p (fig. 4A). In addition, circ\_0007364 transfection could significantly decreased miR-665 and miR-356, but did not affect miR-942-5p (fig. 4B and fig. 4C). Therefore, we chose miR-665 as circ\_0007364 target miRNA for further study. We constructed wild type circ\_0007364 containing complementary sites of miR-665 (WT-circ\_0007364) and mutant circ\_0007364 containing mutation sites of miR-665 (MUT-circ\_0007364) (fig. 4D) and overexpressing vector of miR-665 (fig. 4E) was also obtained. The results showed that when WT-circ\_0007364 combined with miR-665, relative luciferase activity decreased significantly, while MUT-circ\_0007364 and miR-665 were transfected into cells had no significant effect on relative luciferase activity (fig. 4F and fig. 4G). Moreover, miR-665 was significantly down regulated in CC tissues and cells (fig. 4H and fig. 4I), and had negative correlation with circ\_0007364 in CC tissues (fig. 4J). In addition, inhibition of circ\_0007364 sharply decreased miR-665 expression in CC cells (fig. 4K). Circ\_0007364 silencing reduced miR-665 expression in CC

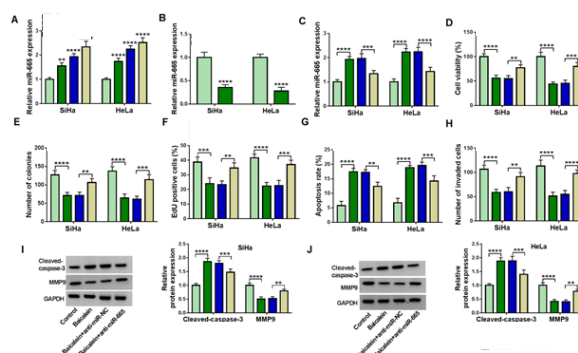
cells (fig. 4L). Moreover, miR-665 expression in circ\_0007364 groups was significantly lower in circ\_0007364 groups compared with that in pCD5-miR group. Therefore, circ\_0007364 bound to miR-665 and regulated its expression in CC cells.

We measured the expression of miR-665 in CC cells under different concentrations to understand the relationship between miR-665 and baicalein. MiR-665 was gradually increased with the increase of baicalein concentration (fig. 5A). Therefore, we transfected the oligos of anti-miR-665 into CC cells and anti-miR-665 transfection inhibited the expression of miR-665 (fig. 5B). Less than 20  $\mu$ g baicalein, inhibition of miR-665 could weaken miR-665 expression induced by baicalein (fig. 5C). Moreover, inhibiting the expression of miR-665 weakened baicalein-mediated inhibition in cell viability (fig. 5D), cell clone number (fig. 5E), EdU positive cells (fig. 5F) and invasion (fig. 5G) and the promotion of apoptosis (fig. 5H). Additionally, baicalein increased cleaved-caspase-3 production and decreased MMP9 expression in CC cells and these phenomena were reversed through down regulating miR-665 (fig. 5I and fig. 5J). Therefore, inhibiting miR-665 expression could weaken baicalein-induced effects in CC cells.



**Fig. 4:** Circ\_0007364 interacted with miR-665, (A): Prediction analysis of target miRNA of circ\_0007364; (B and C): MR-665, miR-346 and miR-942-5p was detected in pCDS-ciR and circ\_0007364 groups in CC cells via qRT-PCR; (D): Binding sites between miR-665 and circ\_0007364; (E): qRT-PCR analysis of miR-665 content in CC cells; (F and G): Association analysis of miR-665 with circ\_0007364; (H): qRT-PCR analysis of miR-665 expression; (I): Relationship between miR-665 and circ\_0007364 was examined using Pearson correlation analysis in tumor tissues; (J): qRT-PCR analysis of miR-665 expression; (K): Analysis of circ\_0007364 expression efficiency si-NC via qRT-PCR and (L): MiR-665 content was assessed in si-NC, si-circ\_0007364, pCD5-ciR and circ\_0007364 groups of CC cells using qRT-PCR

Note: (B) ( ): pCDS-ciR and ( ): Circ\_0007364; (E) ( ): miR-NC and ( ): miR-665; (K) ( ): si-NC and ( ): si-circ\_0007364 and (L) ( ): pCDS-ciR and ( ): circ\_0007364



**Fig. 5:** miR-665 inhibitor reversed baicalein-mediated proliferation, apoptosis and invasion in CC cells. (A): MiR-665 content was assessed under different concentration baicalein (control, 10, 20, 30 µg/ml) in CC cells; (B): Analysis of miR-665 expression efficiency using qRT-PCR; (C): Baicalein and miR-665 down regulation-mediated effect on miR-665 expression; (D to F) Cell proliferation in control, baicalein, baicalein+anti-miR-NC and baicalein+anti-miR-665 groups of CC cells; (G): Cell apoptosis in control, baicalein, baicalein+anti-miR-NC and baicalein+anti-miR-665 groups of CC cells; (H): Cell invasion in each groups and (I and J): Cleaved-caspase-3 and MMP expression was measured in each groups using Western blot assay

Note: (A) ( ): Control; ( ): 10 µg/ml; ( ): 20 µg/ml and ( ): 30 µg/ml; (B) ( ): anti-miR-NC and ( ): anti-miR-665 and (C) ( ): Control; ( ): baicalein; ( ): baicalein+anti-miR-NC and ( ): baicalein+anti-miR-665

The target gene of miR-665 through Star base 2.0. TPD52 contained binding sites with miR-665 (fig. 6A). In addition, when WT-TPD52 3' UTR rather than MUT-TPD52 3' UTR was transfected with miR-665, the luciferase activity decreased significantly (fig. 6B and fig. 6C). Moreover, the network database TCGA showed TPD52 was highly expressed in CC tissues (fig. 6D and fig. 6E). In our experiment, we also found a high TPD52 expression in CC tissues and cells (fig. 6F-fig. 6I) and TPD52 was negatively correlated with miR-665 (fig. 6J). Additionally, miR-665 negatively regulated TPD52 expression. Therefore, these results suggested miR-665 directly bound to TPD52 in CC cells.

Next, TPD52 content was assessed in CC cells after baicalein treatment. The results showed that TPD52 level in cancer cells was decreased with increasing baicalein concentration (fig. 7A). We transfected the overexpression vector of TPD52 into CC cells to clarify the relationship between TPD52 and baicalein (fig. 7B). Increasing the expression of TPD52 can restore the inhibitory effect of baicalein on TPD52 expression (fig. 7C). Moreover, baicalein significantly inhibited cell proliferation (fig. 7D-fig. 7F) and invasion (fig. 7G), induced apoptosis (fig. 7H), while overexpression of TPD52 attenuated these effects of baicalein on the progression of CC cells. Increasing TPD52 expression reversed the expression of cleaved-caspase-3 and MMP9 affected by baicalein (fig. 7I and fig. 7J). Therefore, overexpression of TPD52 weakened baicalein induced effects in CC cells.

Next, we found that si-circ\_0007364 transfection

not only inhibited the expression of TPD52 (fig. 8A), cell proliferation (fig. 8B-fig. 8D), cell invasion (fig. 8E) and the protein expression of MMP9 (fig. 8F and fig. 8G), but also induced apoptosis (fig. 8H) and cleaved-caspase-3 production (fig. 8G and fig. 8H), while overexpression of TPD52 reversed these effects. Additionally, inhibition of miR-665 improved TPD52 expression decreased by si-circ\_0007364 transfection (fig. 9A). Therefore, circ\_0007364 regulated the expression of TPD52 by binding miR-665 and affected cell proliferation invasion and apoptosis in CC cells. Moreover, under baicalein treatment, promotion of circ\_0007364 or inhibition of miR-665 significantly induced TPD52 expression that was inhibited by baicalein (fig. 9B and fig. 9C). In conclusion, baicalein inhibited the growth of CC cells by affecting circ\_0007364/miR-665/TPD52 axis.

Mouse model assay was used to determine circ\_0007364 *in vivo* and the results showed that circ\_0007364 expression was sharply decreased in sh-circ\_0007364 group (fig. 10A). After 8 d, mice were injected with baicalein (45 mg/kg) with sh-NC or sh-circ\_0007364, once every 3 d. As shown in fig. 10B and fig. 10C, circ\_0007364 silencing inhibited tumor volume and weight, and sh-circ\_0007364 combined with baicalein could further inhibit tumor growth. In addition, baicalein also induced miR-665 expression that was promoted by sh-circ\_0007364 and inhibited circ\_0007364 and TPD52 expression that was suppressed by sh-circ\_0007364 (fig. 10D-fig. 10F). Therefore, knockdown of circ\_0007364

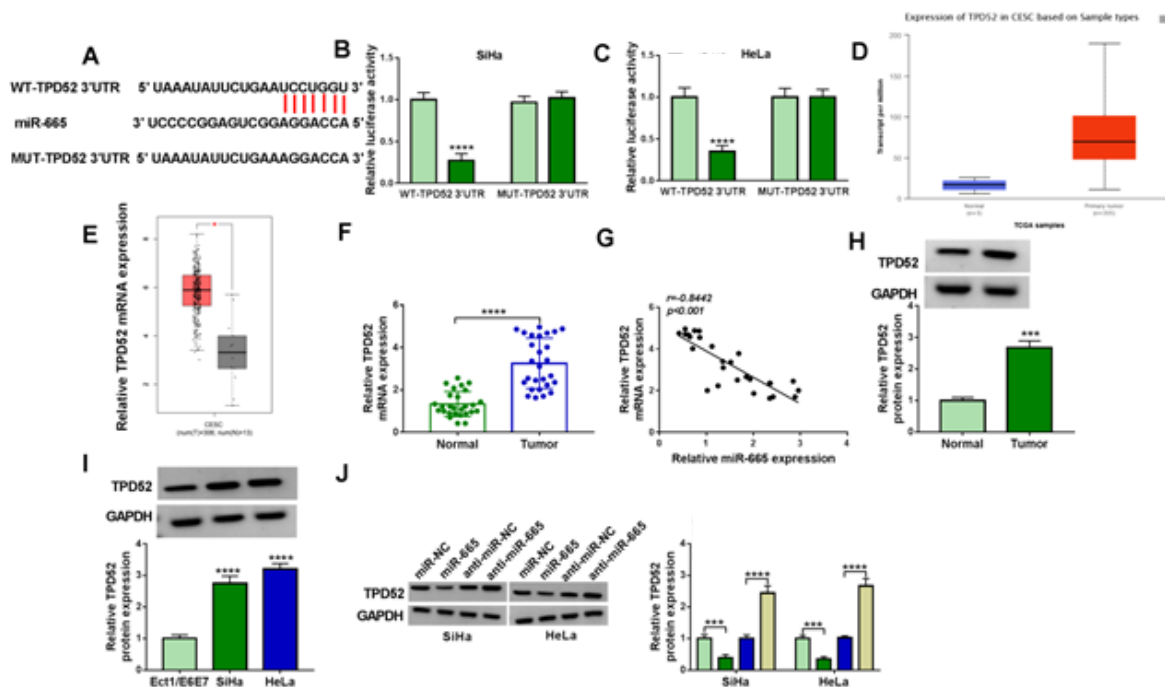


Fig. 6: miR-665 directly interacted with TPD52, (A): Binding sites between miR-665 and TPD52; (B and C): Luciferase activity was detected in CC cells transfected with miR-665 and WT-TPD52 3'UTR groups; (D and E): TPD52 expression analysis through TCGA and CESC database; (F): TPD52 expression in tumor tissues and normal tissues; (G): Relationship between miR-665 and TPD52 in tumor tissues; (H and I): TPD52 was detected in CC tissues or cells and normal tissues or cells and (J): MiR-665-mediated effect on TPD52 expression

Note: (B) ( ■ ): miR-NC and ( ■ ): miR-665 and (J) ( ■ ): miR-NC; ( ■ ): miR-665; ( ■ ): Anti-miR-NC and ( ■ ): Anti-miR-665

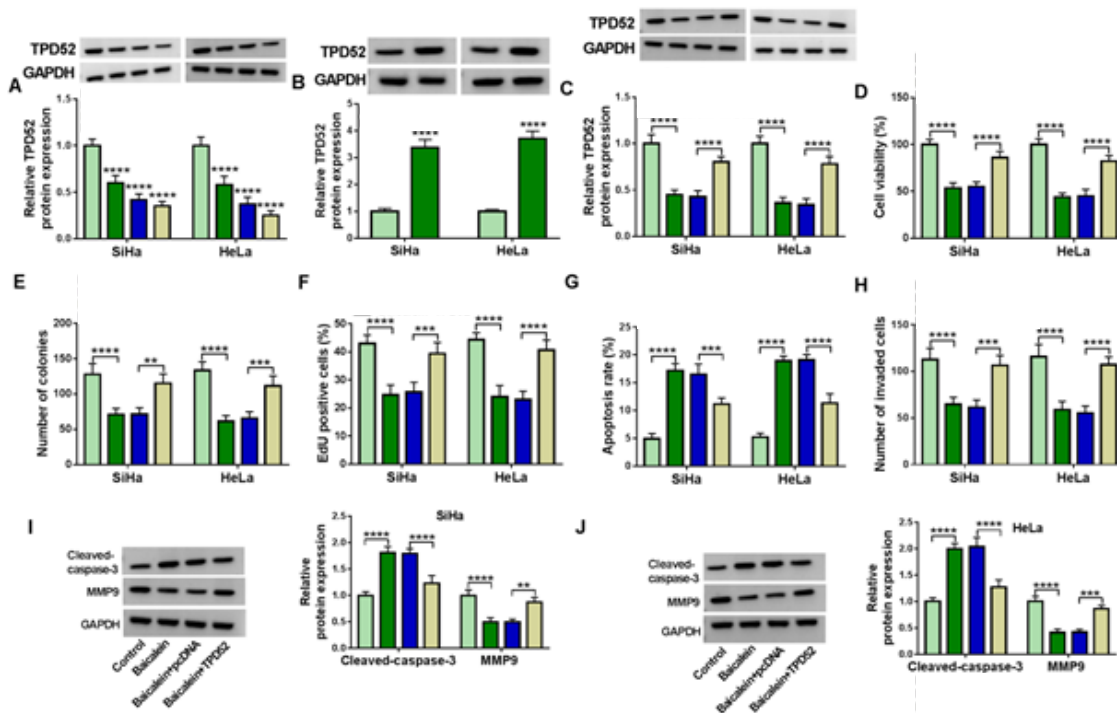


Fig. 7: TPD52 suppressed baicalein-mediated CC cell apoptosis and promoted baicalein-mediated proliferation and invasion, (A): TPD52 expression was detected under different concentration baicalein (control, 10, 20, 30 µg/ml) in CC cells; (B): Analysis of TPD52 expression in CC cells transfected with TPD52 and pcDNA; (C): TPD52 was detected in control, baicalein, baicalein+pcDNA and baicalein+TPD52 groups of CC cells; (D-F): Cell viability was detected in control, baicalein, baicalein+pcDNA and baicalein+TPD52 groups of CC cells; (G): Cell apoptosis was detected in control, baicalein, baicalein+pcDNA and baicalein+TPD52 groups of CC cells; (H): Cell invasion was measured in CC cells in control, baicalein, baicalein+pcDNA and baicalein+TPD52 groups of CC cells using Transwell assay and (I and J): Cleaved-caspase-3 and MMP expression were measured in control, baicalein, baicalein+pcDNA and baicalein+TPD52 groups of CC cells

Note: (A) ( ■ ): Control; ( ■ ): 10 µg/ml; ( ■ ): 20 µg/ml and ( ■ ): 30 µg/ml; (B) ( ■ ): pcDNA and ( ■ ): TPD52 and (C) ( ■ ): Control; ( ■ ): Baicalein; ( ■ ): Baicalein+pcDNA and ( ■ ): Baicalein+TPD52



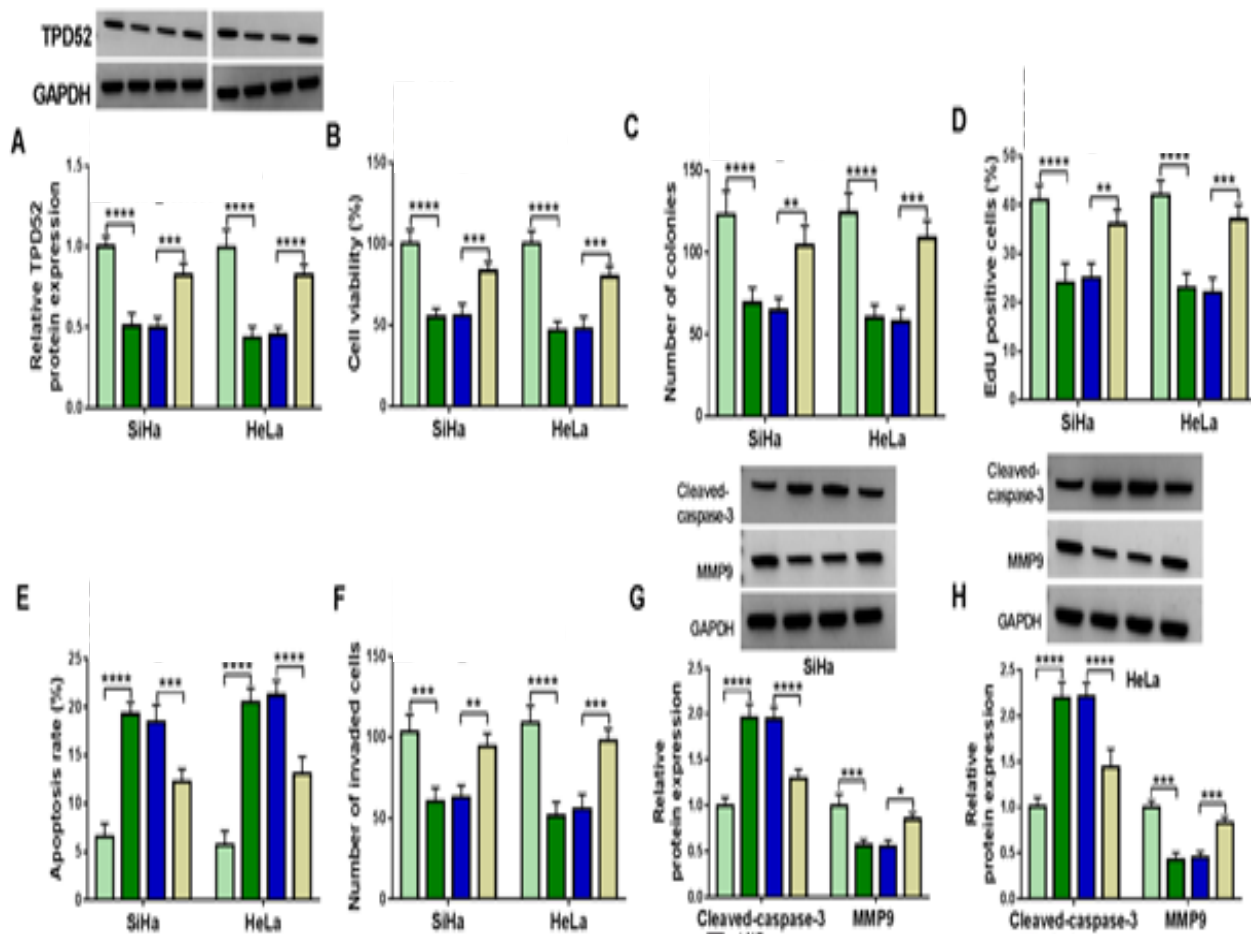


Fig. 8: TPD52 reversed si-circ\_0007364-mediated effects on CC cell progression. CC cells were divided into si-NC, si-circ\_0007364, si-circ\_0007364+pcDNA and si-circ\_0007364+TPD52 groups, (A): Analysis of TPD52 expression; (B to D): Cell viability was detected; (E) Cell apoptosis was detected; (F) Cell invasion was measured using Transwell assay and (G and H): Cleaved-caspase-3 and MMP protein expression analysis  
Note: ( ■ ): si-NC; ( ■ ): si-circ-0007364; ( ■ ): si-circ-0007364+pcDNA and ( ■ ): si-circ-0007364+TPD52

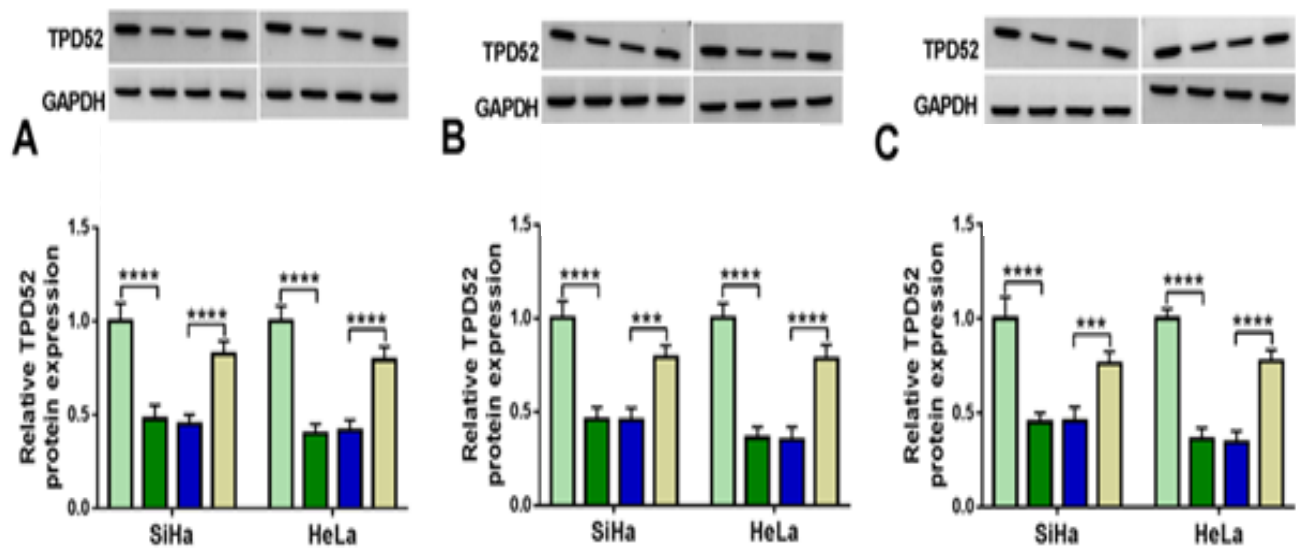
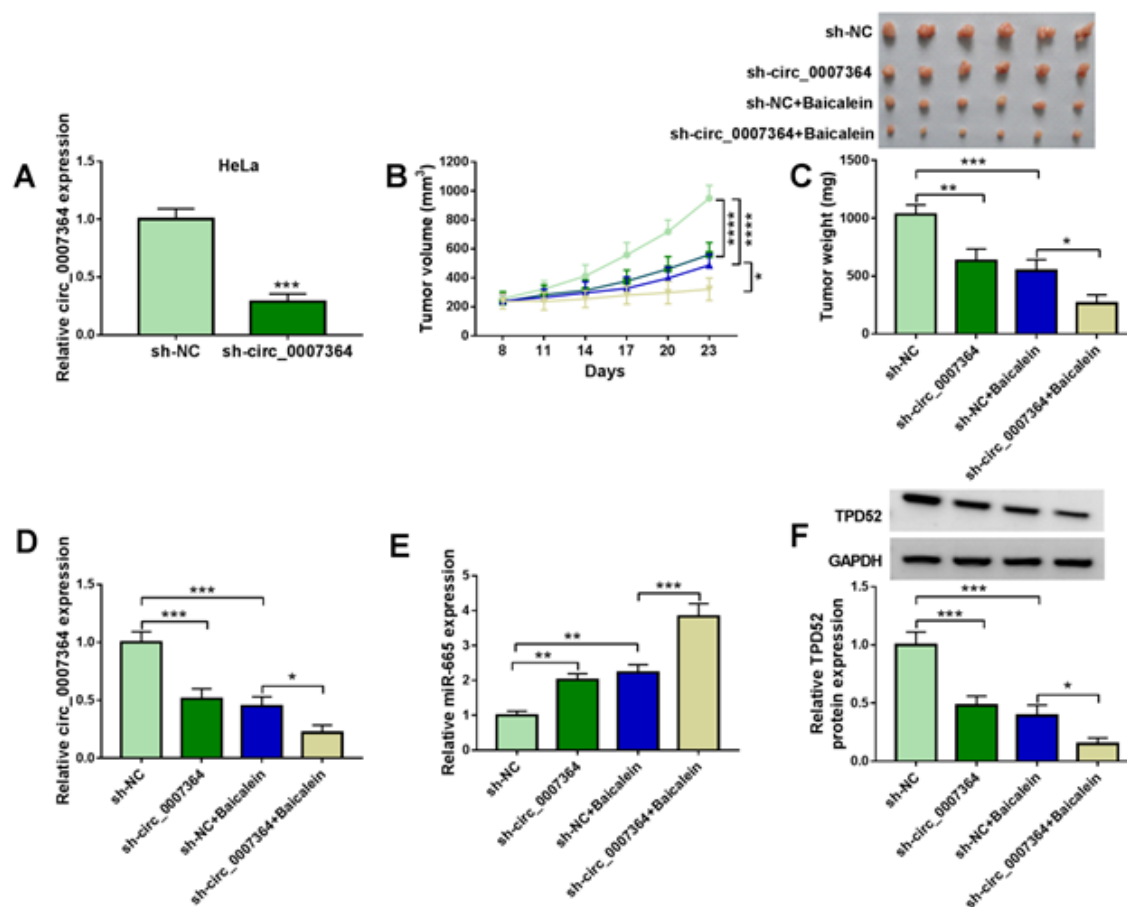


Fig. 9: Baicalein affected circ\_0007364/miR-665/TPD52 axis, (A): TPD52 was expression analysis after circ\_0007364 silencing and miR-665 down regulation; (B): TPD52 expression in control, baicalein, baicalein+pcD5-miR and baicalein+circ\_0007364 groups of CC cells and (C): TPD52 expression in control, baicalein, baicalein+anti-miR-NC and baicalein+anti-miR-665 groups of CC cells  
Note: (A): ( ■ ): si-NC; ( ■ ): si-circ\_0007364; ( ■ ): si-circ\_0007364+anti-miR-NC and ( ■ ): si-circ\_0007364-miR-665 and (B): ( ■ ): Control; ( ■ ): Baicalein; ( ■ ): Baicalein+pcD5-ciR and ( ■ ): Baicalein+anti-miR-NC



**Fig. 10:** Knockdown of circ\_0007364 improved baicalein anti-cancer effect *in vivo*, (A): Sh-circ\_0007364 transfection inhibited circ\_0007364 expression; (B and C): Tumor volume and weight were measured after circ\_0007364 silencing and baicalein treatment and (D-F): Circ\_0007364, miR-665 and TPD52 expression analysis in each group was detected in sh-NC, sh-circ\_0007364, sh-NC+baicalein and sh-circ\_0007364+baicalein groups *in vivo*

Note: ( — ): sh-NC; ( — ): sh-circ\_0007364; ( — ): sh-NC+baicalein and ( — ): sh-circ\_0007364+baicalein

increased baicalein anti-cancer effect *in vivo*.

Nowadays, disease-related databases are constantly improving with the development of technology, providing high-quality resources for the study of disease mechanism. Traditional Chinese medicine is remarkably effective in treating diseases<sup>[23-26]</sup>. Baicalein is the main active component of *Scutellaria baicalensis* and has inhibitory effects on cancer progression<sup>[27,28]</sup>. However, the specific regulation mechanism is not perfect.

In CC, circRNA/miRNA/mRNA is one of the important regulatory pathways. Circ\_0000515/miR-326/ELK1 axis was involved in CC progression<sup>[29]</sup>. In addition, circ\_Nuclear Receptor-Interacting Protein 1 (NRIP1) enhanced cell invasion through competitively combining miR-629-3p to regulate PTP4A1/ERK1/2 pathway in CC<sup>[30]</sup>. CircRNA is also involved in the inhibitory mechanism of baicalein in CC development. For

example, baicalein inhibited CC cells growth through regulation of circHIAT1/miR-19a-3p pathway<sup>[9]</sup>. In our study, circ\_0007364 was up regulated in CC tissue samples and baicalein inhibited circ\_0007364 expression. Thus, we speculated that circ\_0007364 participated in the inhibition mechanism of baicalein in CC. Otherwise, circ\_0007364 is derived from PTP4A2, which encodes PTP4A2 protein and belongs to class of protein tyrosine phosphatase family<sup>[31]</sup>. PTP4A2 can promote the production of breast tumor cells<sup>[32,33]</sup>. Previous articles have studied that circ\_0007364 was highly expressed in CC. Moreover, the increased circ\_0007364 expression affected CC progression *via* adjusting miR-101-5p/MAT2A axis<sup>[22]</sup>. Our study showed upregulation of circ\_0007364 could suppress baicalein inhibited cell progression in CC cells.

MiRNA is a noncoding small RNA, which mediates the biological process of cells<sup>[34,35]</sup>. Moreover,

miRNA negatively regulates gene expression by directly degrading target mRNA<sup>[36]</sup>. In this paper, miR-665 was the target miRNA of circ\_0007364. In previous studies, miR-665 can inhibit or promote cancer progression in many cancers, like ovarian cancer, HCC, pancreatic cancer and CC<sup>[37-40]</sup>. In this paper, miR-665 expression was down regulated in CC tissues and cell lines, and baicalein promoted miR-665 expression. Function experiment showed that baicalein regulated CC progression through regulating circ\_0007364 to sponge miR-665 in CC.

Moreover, TPD52 was determined to bind to miR-665 in CC and was associated with the regulatory pathway of tumor inhibited by baicalein through dual luciferase report assay and function experiment. Various study demonstrated that TPD52, act as cancer-oncogene, participated in cancer development, which are upregulated in many cancer types, including CC<sup>[41-44]</sup>. Consistent with previous study<sup>[44]</sup>, TPD52 was up regulated in CC cells and was related to cell progression in CC in the present work. Furthermore, baicalein reduced TPD52 expression in CC cells. Thus, we determined that baicalein inhibited CC development through modulating circ\_0007364/miR-665/TPD52 axis.

This study described how baicalein inhibited the regulatory pathway of CC cell development. Baicalein regulated the growth of CC cells by affecting circ\_0007364/miR-665/TPD52 axis, providing a new molecular mechanism of baicalein in CC and finding the key regulatory marker. This exploration could further improve the medication of baicalein and played a better anti-cancer effect. At the same time, this investigation could also be used as a research idea to find the molecular mechanism of other anticancer drugs, and find more effective anticancer drug components and contribute to cancer treatment.

#### Author's contributions:

Xuefang Liu and Jing Xie have contributed equally to this work.

#### Conflict of interests:

The authors declared no conflict of interests.

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