

Curcumin carried by polystyrene nanoparticles inhibits phosphatidylinositol 3-kinase/protein kinase B signaling pathway through miR-186 and down-regulates Twist to inhibit the epithelial-mesenchymal transition in colorectal cancer in mice

Peinan Wen¹, Mingshu Lin¹ and Jicai Chen^{2*}

¹Department of Anorectal Surgery, Pingyang Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, 325499, China

²Wenzhou Medical University Affiliated First Hospital, Wenzhou, Zhejiang, 325035, China

Abstract: Background: Epithelial-mesenchymal transition (EMT) is involved in colorectal cancer (CRC) pathogenesis. Curcumin (CUR) is widely used in the treatment of CRC. Polystyrene nanoparticles (PS-NPS) have a high specific affinity and have an inhibitory effect on cell proliferation. Therefore, the purpose of this study is to investigate the mechanism of inhibition of EMT in colorectal cancer mice by preparing curcumin polystyrene nanoparticles (CUR-PS-NPS). **Objective:** This study aimed to construct CUR-PS-NPS and investigate their mechanism in inhibiting CRC EMT via the miR-186/PI3K/AKT/ Twist axis. **Method:** CUR-PS-NPS were prepared and characterized. In-vivo, CRC model mice (Balb/c, male) were divided into four groups: sham-operated control (PC), CRC model (CRC), low-dose CUR-PS-NPS (Cur-PS-NPS-L), and high-dose CUR-PS-NPS (Cur-PS-NPS-H). After intraperitoneal injection, tumor volume, EMT marker proteins and changes in miR-186 and the PI3K/AKT/ Twist pathway were measured. In-vitro, human normal colonic epithelial cells (NCM460) were used to validate miR-186 interference and SW480 cells were co-transfected with a PI3K inhibitor to clarify the specific mechanism by which CUR-PS-NPS inhibit EMT. **Results:** In CRC mice intervened with CUR-PS-NPS, the tumor volume was reduced by 50%, the expression of miR-186 was up-regulated by 2.5 times, the phosphorylation level of PI3K/AKT was decreased by 40%, the expression of Twist protein was down-regulated by 35% and the expression of the EMT marker E-cadherin was increased. The expressions of N-cadherin, Vimentin and Snail decreased significantly. **Conclusion:** CUR-PS-NPS can effectively delay the EMT process of CRC by up-regulating miR-186, inhibiting the PI3K/AKT signaling pathway and the expression of Twist. This discovery provides a new strategy for targeted therapy based on nanocarriers and its application potential in clinical practice can be further explored in the future.

Keywords: Curcumin; Colorectal cancer; Epithelial-mesenchymal transition; Microrna-186; Polystyrene nanoparticles

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INTRODUCTION

Colorectal Cancer (CRC) is a leading cause of death among cancer populations (Baidoun *et al.*, 2024). Chemotherapy is still the main treatment for CRC at present. Still, the specificity of chemotherapy is low, which not only causes side effects on the body and reduce the effectiveness of the treatment, but also lead to serious drug resistance (Islam *et al.*, 2022). Studies have shown (Zhang *et al.*, 2021). During the treatment of CRC, epithelial-to-mesenchymal transition (EMT) and basic helix-loop-helix (bHLH) transcription factor Twist of tumor cells regulate tumor cell sensitivity to chemotherapeutic drugs. EMT is critical for the metastasis of colorectal cancer. During the EMT process, the morphology and phenotype of epithelial tumor cells undergo significant changes, including loss of cell adhesion, reorganization of cell polarity and cytoskeleton, which makes the cells more invasive and phenotypic and this has the effect of promoting immunosuppression (Taki *et al.*, 2021; Basak *et al.*, 2023). The Twist family consists of Twist1, Twist2, etc., which are the main factors that activate EMT transcription factors (Kciuk *et al.*, 2022).

Twist can promote tumor cell abilities, endowing them with stem cell-like properties and treatment resistance (Greco *et al.*, 2021; Liu *et al.*, 2023). Although the early diagnosis of cancer and the continuous optimization of treatment have reduced the mortality rate, the resistance of the body to therapeutic drugs makes an urgent need to explore new CRC treatments that can target colorectal cancer and effectively inhibit EMT.

Curcumin (Cur) is the main component of turmeric, a traditional medicinal plant in china. It has been proven to have good therapeutic effects in many chronic diseases and cancers (Hu *et al.*, 2023). Its anticancer mechanism mainly includes inhibiting tumor proliferation, invasion and metastasis, regulating apoptosis and autophagy (Kabir *et al.*, 2021; Ming *et al.*, 2022). Cur can affect triple-negative breast cancer (TNBC) cells by inhibiting Hedgehog/Gli signaling activity (Li *et al.*, 2023) to achieve its therapeutic effect on breast cancer. Cur is a drug that can block the cell cycle and proliferation (Li *et al.*, 2021) found that. Cur can reverse N-methyltransferase-induced proliferation and resistance to 5-fluorouracil in CRC cells. However, due to the fast metabolism and poor water solubility of Cur, its

*Corresponding author: e-mail: cjc60321@163.com

efficiency has been limited. The rapid development of nanotechnology in nanomedicine formulations brings great hope to improve the current situation of drug resistance in cancer treatment. Polystyrene nanoparticles (PS-NPs) were selected as the delivery vehicle for curcumin due to their excellent biocompatibility, ease of surface modification, controllable drug release profiles and superior stability. Compared to alternative nanocarriers such as liposomes or PLGA, PS-NPs exhibit a longer circulation half-life and lower immunogenicity, making them suitable for both oral and injectable delivery systems (Huang *et al.*, 2024; Ozturk *et al.*, 2020). As an emerging nanomaterial, PS-NPs can slow down the degradation of drugs. However, PS-NPs carrying curcumin have not been reported. Therefore, Cur was carried onto PS-NPs to explore its role and mechanism in CRC.

MicroRNA (miRNA) plays a regulatory role in metabolism, growth, development and other biological functions (He *et al.*, 2020). miRNA expression dysregulated in cancer, is involved in tumorigenesis and can regulate the progression and development of cancer through the interaction with cancer-related genes (Hill and Tran, 2021). Among them, miR-186 is studied in multiple cancers. (Wang *et al.*, 2021) found that miR-186 level was lower in tumor tissues in Lung Adenocarcinoma (LUAD) and associated with tumor metastasis in LUAD patients. After upregulation of miR-186, it inhibits cell behaviors by targeting and regulating Dicer1. In the study of CRC, Bayat's group (Bayat *et al.*, 2021) found that there is a targeted binding relationship between miR-186-5p and SMAD6/7 and its overexpression can downregulate SMAD6/7 to regulate TGF- β signaling, thereby inhibiting CRC development. TWIST1 is considered to be a highly upregulated proto-oncogene in various human cancers and overexpression of TWIST1 is related to tumor progression (Razzaque and Atfi, 2020). It was found in the research results of breast cancer (Hao *et al.*, 2023) that miR-186 can inhibit tumor proliferation by targeting Twist1. However, whether Cur-PS-NPs can affect rectal cancer by regulating the targeted combination of miR-186 and Twist1 and whether there is a pathway regulation, is still unclear. Based on this, in this study, PS-NPs were used as a curcumin vector for the first time. By targeting and regulating the miR-186/PI3K/AKT/Twist axis to inhibit EMT, not only was the delivery problem of curcumin solved, but also an experimental basis was provided for the targeted treatment of CRC, which has important translational medical value.

MATERIALS AND METHODS

Main experimental reagents

Curcumin (Beijing Zeping Bio); human colorectal cancer cells (SW480 Shane Bio); human normal colonic epithelial cells (NCM460 Vanke Bio); azobisisobutyronitrile (AIBN), polyvinylpyrrolidone (PVP), benzene ethylene (St

Shanghai McLean Biochemical Technology); nitrogen (N2 Shanghai Tonghui Special Gas); TWIST1 antibody (Abmart); overexpression of miR-186 (miR-186 mimics), interference with miR-186 (miR-186 inhibitor); PI3K inhibitor LY294002 (Catalog no. L9908, Sigma-Aldrich) PI3K agonist 740Y-P (Item number SML0865, Sigma-Aldrich); TWIST1 antibody (Item number T5693, Abmart) p-PI3K (Item number #4228, CST); p-AKT (Item No. #4060, CST).

Preparation and characterization of curcumin polystyrene nanoparticles

Cur (20 mg/mL) and PEG-PTMC (20 mg/mL) were dissolved with acetone as the organic phase and PVP k90 (2 mg/mL) in water for the aqueous phase. After the system was uniformly stirred, nitrogen gas was introduced to degas it for 10–15 minutes. Subsequently, 0.318 g of AIBN was dissolved in 31.76 g of styrene monomer and slowly added to the reaction system. After that, divinylbenzene and absolute ethanol were mixed. Then, the mixed solution was added at a constant dripping rate (0.5 mL/min) to form an emulsified sample and polymerization reaction was carried out for 24h at 70°C. The nanoparticles were separated and washed three times with deionized water. The morphology and particle size distribution of Cur-PS-NPs were characterized using transmission electron microscopy (TEM, JEOL JEM-1400). Their hydrodynamic diameter and polydispersity index (PDI) were determined by dynamic light scattering (DLS) and their surface charge (zeta potential) was measured using a Zetasizer Nano ZS (Malvern Instruments). All measurements were performed in triplicate.

Experimental animals, grouping, randomization, blinding, and sample size determination

Twenty-four 4-week-old Balb/c male nude mice, weighing 22 $\pm$ 2 g, were purchased from Chengdu Dashuo Experimental Animal Company. After acclimation for one week, all mice were housed in standard clean cages at 22 $\pm$ 2°C and 60% humidity, with ad libitum access to standard feed and sterile drinking water. A 12-hour light/12-hour dark cycle was maintained (lights on from 8:00 AM to 8:00 PM, illuminance 150–200 lux). Bedding consisted of autoclaved corncob material, changed twice weekly. Environmental enrichment (nesting material and red plastic shelters) was provided in all cages.

All experimental procedures were performed during the light phase. A total of 5 $\times$ 10⁶ SW480 cells were inoculated subcutaneously into the left axilla of each mouse, except for those in the paracancerous (PC) group, which received no tumor inoculation and served as normal controls. The successful establishment of the CRC model was defined as tumor volume reaching 90–110 mm³ at 7 days post-inoculation, with typical tumor signals (hyperintensity on T2WI and restricted diffusion on DWI) confirmed by magnetic resonance colonoscopy (MRC).

Table 1: Design of animal experiment schemes

Group	n	Intervention	Route of administration
Sham-operated group (PC)	6	Normal saline	Intraperitoneal injection
CRC Model group	6	Normal saline	Intraperitoneal injection
Low-dose Cur-PS-NPs group (L)	6	Cur 100 mg/kg + PS-NPs 200 ppm	Intraperitoneal injection
High-dose Cur-PS-NPs group (H)	6	Cur 100 mg/kg + PS-NPs 400 ppm	Intraperitoneal injection

Table 2: Real-time PCR primers and primer sequences.

	Primer	Sequences
miR-186	Forward primer	5'-CCCGATAAAGCTAGATAACC-3'
	Reverse primer	5'-CAGT GCGTGTCTGGAGT-3'
TWIST	Forward primer	5'-CGGGAGTCCGCAGTCTTA-3'
	Reverse primer	5'-GCTTGAGGGTCTGAATCTTG-3'
GAPDH	Forward primer	5'-AGGTCATCCCAGAGCTGAACG-3'
	Reverse primer	5'-CACCTGTGCTGTAGCCGTAT-3'

At 7 days post-inoculation, after confirmation of successful tumor engraftment, CRC model mice (n=18) were randomly assigned to three treatment groups (n=6 per group) using a computer-generated random number table: CRC model group (CRC), low-dose Cur-PS-NPs group (Cur-PS-NPs-L), and high-dose Cur-PS-NPs group (Cur-PS-NPs-H). The PC group (n=6) was housed separately without randomization, serving as the normal control group. The randomization sequence was generated by an investigator not involved in subsequent procedures and sealed in opaque envelopes. Group allocation was revealed by this investigator just before the first intervention, and cages were labeled accordingly. Sample size was determined based on pilot data (control vs. Cur-PS-NPs-H: mean tumor volumes 180 mm³ vs. 90 mm³, pooled SD 60 mm³). Using a two-sided $\alpha=0.05$ and power=0.80 for an independent-samples t-test, 5.6 mice per group were required; thus 6 mice per group were used to account for potential attrition. To minimize bias, the investigator responsible for administering intraperitoneal injections was blinded to group allocation. Outcome assessments, including tumor volume measurement by MRC, Western blot quantification, immunohistochemical scoring, and TUNEL analysis, were performed by an independent investigator blinded to treatment groups.

Inclusion criteria for CRC model mice were tumor volume of 90–110 mm³ at 7 days post-inoculation with typical MRC signals. Predefined exclusion criteria were: (1) failure to achieve tumor volume ≥ 90 mm³ or stable tumor at 7 days; (2) severe illness or infection requiring early termination; (3) accidental death. No animals were excluded from analysis. No data points were excluded from any assay, and no statistical outliers were detected by Grubbs' test ($\alpha=0.05$).

Mice in the CRC group received intraperitoneal injections of normal saline, while those in the Cur-PS-NPs-L and Cur-PS-NPs-H groups received low-dose and high-dose Cur-PS-NPs, respectively. Injections were administered once per day with a one-day interval, for a total of seven

injections. Tumor growth was monitored throughout the experiment.

Primary outcome measures were: (1) tumor volume; (2) EMT marker protein expression (E-cadherin, N-cadherin, Vimentin, Snail); (3) miR-186 expression; (4) p-PI3K, p-AKT, and Twist expression. Secondary outcome measures included apoptosis rate, MMP2/MMP9, cell proliferation (MTT, EdU), migration/invasion (Transwell), and Bcl-2 expression.

Anesthesia and analgesia: Surgical procedures (tumor cell inoculation and final tissue collection) were performed under isoflurane inhalation anesthesia (induction 3%, maintenance 1.5–2% in 100% O₂ at 1 L/min). Anesthesia depth was confirmed by pedal withdrawal reflex and respiratory rate. Postoperatively, animals were placed on a warming pad (37°C) until fully recovered. Analgesia was provided with buprenorphine (0.05–0.1 mg/kg, s.c., every 8–12 h for 48 h).

Measures to minimize suffering: (1) All procedures were performed under anesthesia; (2) clinical health assessments were performed twice daily (9:00 AM and 4:00 PM), monitoring body weight, behavior, physical appearance, and signs of distress; (3) humane endpoints were tumor volume >2000 mm³, weight loss >20%, tumor ulceration/bleeding, severe abdominal distension, or persistent severe distress; any animal reaching these endpoints would be immediately euthanized; (4) environmental enrichment (nesting material and shelters) was provided.

Euthanasia: At the endpoint, all animals were deeply anesthetized with isoflurane (5%, until respiratory arrest) followed by cervical dislocation. No animals reached humane endpoints prematurely.

Handling of missing data and sample size for each analysis: No animal deaths or sample loss occurred during this study. All 24 enrolled mice (n=6 per group; 18 CRC model mice and 6 PC controls) completed the experiment and were included in the analysis. For all histological and molecular

biological assays, including Western blot, qRT-PCR, immunohistochemistry, and TUNEL staining, tumor tissue samples from all 6 mice in each group were used, with three technical replicates per sample. For in vitro experiments, all experiments were independently repeated three times, with three replicates per group in each experiment. No data points were excluded from the final analysis. Therefore, the sample sizes for each outcome measure were: in vivo experiments, n=6 mice per group; in vitro experiments, n=3 independent replicates.

Cell culture and treatment

The culture conditions of cells SW480 and NCM460 were RPMI1640 + 10% fetal bovine serum + 1% penicillin/streptomycin. NCM460 cells in the logarithmic growth phase were taken for nano-intervention and transfection experiments. The cells were grouped as follows: Cur-PS-NPs + miR-186 NC group, Cur-PS-NPs + miR-186 inhibitor group, Cur-PS-NPs + 740Y-P group, Cur-PS-NPs + miR-186 inhibitor + Group LY294002. SW480 cells were set as the Control group and NCM460 cells were set as the NC group.

Experimental method

Magnetic resonance colonoscopy (MRC) confirms the successful establishment of the colorectal cancer mouse model (Zhang et al., 2023)

Wash the enema with 150 μ L of normal saline first. Since the colorectum does not produce MR proton signals, the colorectum is filled with fluoride. Fat-suppressed T1-weighted images (T1w) were acquired using a 9.4 T MRI scanner before tumor cell implantation, 7 days, 14 days and 21 days. T1w coronal MR parameters are: TR/TE, 1000/8.99 ms; field of view, 50 $\times$ 40 mm; matrix, 192 $\times$ 128.

Immunohistochemical detection (Hung and Chirieac, 2024)

After washing with PBS, it was blocked in 3% H₂O₂ for 10 min, then boiled in EDTA buffer for 20 min, incubated with bovine serum albumin for 20 minutes and then used with antibodies against Ki67 or MMP9 at 4 $^{\circ}$ C. Incubated overnight at $^{\circ}$ C, then incubated with enzyme-labeled secondary antibody and stained with DAB.

TUNEL staining (Rossello and Adell, 2023)

Tumor tissue sections were dewaxed and rehydrated, then incubated with 15 μ g/mL proteinase K at 37 $^{\circ}$ C. After rinsing, the sections were incubated in equilibration buffer. A mixture containing 50 μ L of Biotin-dUTP Labeling Mix and 3 μ L of TdT enzyme was then added and incubated for 1 hour. The reaction was terminated by incubating the sections in stop buffer for 10 minutes (0.17 hours), followed by three washes. The specimens were subsequently covered with streptavidin-HRP, washed again, visualized with DAB substrate, and observed under a microscope.

Western blot

The protein concentration was determined by the BCA method. After preparing the gel, 20 μ g of protein samples

were loaded and transferred after 10% SDS-PAGE electrophoresis. Seal the 5% skimmed milk at room temperature for 1 hour and add the primary antibodies (p-PI3K 1:1000, p-AKT 1:2000, E-cadherin 1:1000, N-cadherin 1:1000, Vimentin 1:2000, β -actin 1:5000). Incubate at 4 $^{\circ}$ C overnight. TBST washed the membrane three times and HRP-labeled secondary antibody (1:5000) was incubated at room temperature for 2 hours, with β -actin as the internal reference. ECL development. Taking β -actin as the internal reference, the gray values of Western blot bands were quantitatively analyzed using ImageJ (NIH) software. The relative expression level of the target protein was calculated (the gray value of the target protein band/the gray value of the β -actin band in the corresponding lane) and expressed as a multiple change relative to the control group. All experiments were independently repeated at least three times.

qRT-PCR

RNA was extracted for cDNA synthesis, followed by PCR using primers in table 1. GAPDH is used as a housekeeping gene. Gene relative level was determined by the $E = 2^{-\Delta\Delta C_t}$ method and the critical threshold period (Ct) value was detected. The specific calculation method is: $\Delta C_t = C_t$ (target gene) - C_t (internal reference gene GAPDH); $\Delta\Delta C_t = \Delta C_t$ (experimental group) - ΔC_t (control group). In this study, the Control group was set as the sham operation group (PC group) or the untreated cell control group. Relative expression level (Fold Change) = $2^{-\Delta\Delta C_t}$. All experiments were set with at least three technical replicates and three biological replicates. The results are expressed as mean $\pm$ standard deviation.

Transwell assay

For cell migration assays, SW480 cells were suspended in serum-free medium. The cell suspension (100 μ L at a density of 10⁶ cells/mL) was then seeded into the upper chambers of 24-well plates. After incubation for 48 hours, the cells were fixed with 4% paraformaldehyde solution and subsequently stained. The number of migrated cells was observed using a magnifying glass. The cell invasion assay method is similar to the cell migration assay method. Before cell inoculation, mix Matrigel was mixed with medium at a ratio of 1:8 and 50 μ L was added the upper chamber. After the Matrigel solidified, cells were seeded in the upper chamber. Other steps were the same as for cell migration experiments.

MTT detection of cell proliferation

After cells were treated according to the experimental protocol, the SW480 cells were inoculated at a density of 5 $\times$ 10³ cells/mL. 10 μ L MTT was added for 4 h and then 150 μ L DMSO. After shaking with a shaker for 10 minutes, the absorbance at 490 nm was measured. After treating SW480 cells with different concentrations of Cur-PS-NPs for 48 hours, a dose-response curve was fitted using GraphPad Prism 8.0.2 software through nonlinear regression and the IC₅₀ value was calculated.

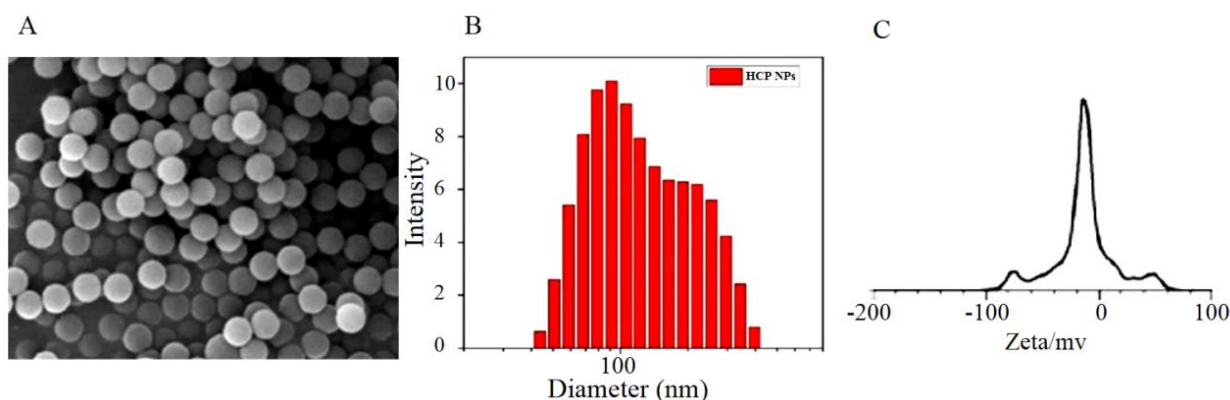


Fig. 1: SEM image and particle size distribution of Cur-PS-NPs. (A) Scanning electron microscope image of Cur-PS-NPs (magnification $\times 50,000$, scale bar: 200 nm); (B) Particle size distribution of Cur-PS-NPs; (C) The Zeta potential of Cur-PS-NPs.

EdU staining

The treated SW480 was incubated with 20 mmol/L EdU for 2 hours. Fix cells with 4% paraformaldehyde for 20 minutes, followed by analysis of EdU-positive cells.

Flow cytometry detection of apoptosis

The treated SW480 was transfected and cultured for 48 hours, digested and made into a 1×10^6 /mL cell suspension, centrifuged, washed, resuspended and then added 5 μ L PI and 10 μ L Annexin V-FITC, incubated for 0.25 h and analyzed by flow cytometry. Apoptosis was observed in different groups.

Statistical method

To minimize bias, mice were randomly assigned to experimental groups using a computer-generated random number sequence. The investigator responsible for administering intraperitoneal injections was blinded to group allocation. Outcome assessments, including tumor volume measurement by MRC, Western blot quantification, immunohistochemical scoring, and TUNEL analysis, were performed by an independent investigator blinded to treatment groups. No animals were excluded from the analysis. Sample size was calculated based on pilot experiment data, which showed mean tumor volumes of 180 mm³ and 90 mm³ in the control and Cur-PS-NPs-H groups, respectively, with a pooled standard deviation of 60 mm³. Using a two-sided $\alpha = 0.05$ and power = 0.80 for an independent-samples t-test, the required sample size was 5.6 mice per group. To compensate for possible animal attrition or modeling failure, 6 mice per group were used. All statistical analyses were conducted using GraphPad Prism 8.0.2.

Handling of missing data and sample size for each analysis: No animal deaths or sample loss occurred during this study. All 24 enrolled mice ($n=6$ per group; 18 CRC model mice and 6 PC controls) completed the experiment and were included in the analysis. For all histological and molecular biological assays, including Western blot, qRT-PCR,

immunohistochemistry, and TUNEL staining, tumor tissue samples from all 6 mice in each group were used, with three technical replicates per sample. For in vitro experiments, all experiments were independently repeated three times, with three replicates per group in each experiment. No data points were excluded from the final analysis. Therefore, the sample sizes for each outcome measure were: in vivo experiments, $n=6$ mice per group; in vitro experiments, $n=3$ independent replicates.

All statistical analyses were performed using GraphPad Prism 8.0.2. For in vivo experiments, $n=6$ mice per group; for in vitro experiments, $n=3$ independent replicates with three technical replicates each. No missing data occurred. Normality of data distribution was assessed using the Shapiro-Wilk test. Homogeneity of variances was assessed using Levene's test. All data met the assumptions of normality and homogeneity of variance ($p > 0.05$), and therefore parametric tests were applied. No data points were excluded, and no statistical outliers were detected by Grubbs' test ($\alpha=0.05$). The data were expressed as mean $\pm$ standard deviation (Mean $\pm$ SD) using GraphPad Prism 8.0.2. Comparisons were performed using one-way analysis of variance (ANOVA). If the results were significant, Tukey's HSD test was applied for post hoc multiple comparisons. Pairwise comparisons between groups were conducted using unpaired t-tests. Effect sizes were calculated using Cohen's d for pairwise comparisons and η^2 for ANOVA. For example, the effect size for tumor volume reduction between CRC and Cur-PS-NPs-H groups was Cohen's $d = 1.82$ (95% CI: 0.78 to 2.86). A p -value < 0.05 was considered statistically significant.

RESULTS

Morphology and particle size distribution of curcumin carried by polystyrene nanoparticles

Transmission electron microscopy revealed that Cur-PS-NPs particles were round and well-dispersed (Fig. 1A); The particle size distribution of Cur-PS-NPs is uniform, with an average particle size of 100 ± 5 nm (Fig. 1B).

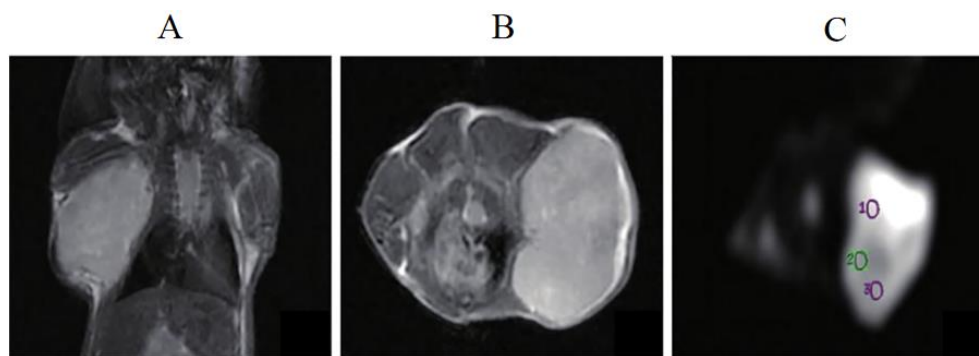


Fig. 2: Tumor volume of colorectal cancer mouse model. (A) The coronal T2WI sequence of the subcutaneous transplanted tumor; (B) The transverse T2WI sequence of the subcutaneous transplanted tumor; (C) The transverse DWI image of the subcutaneous transplanted tumor.

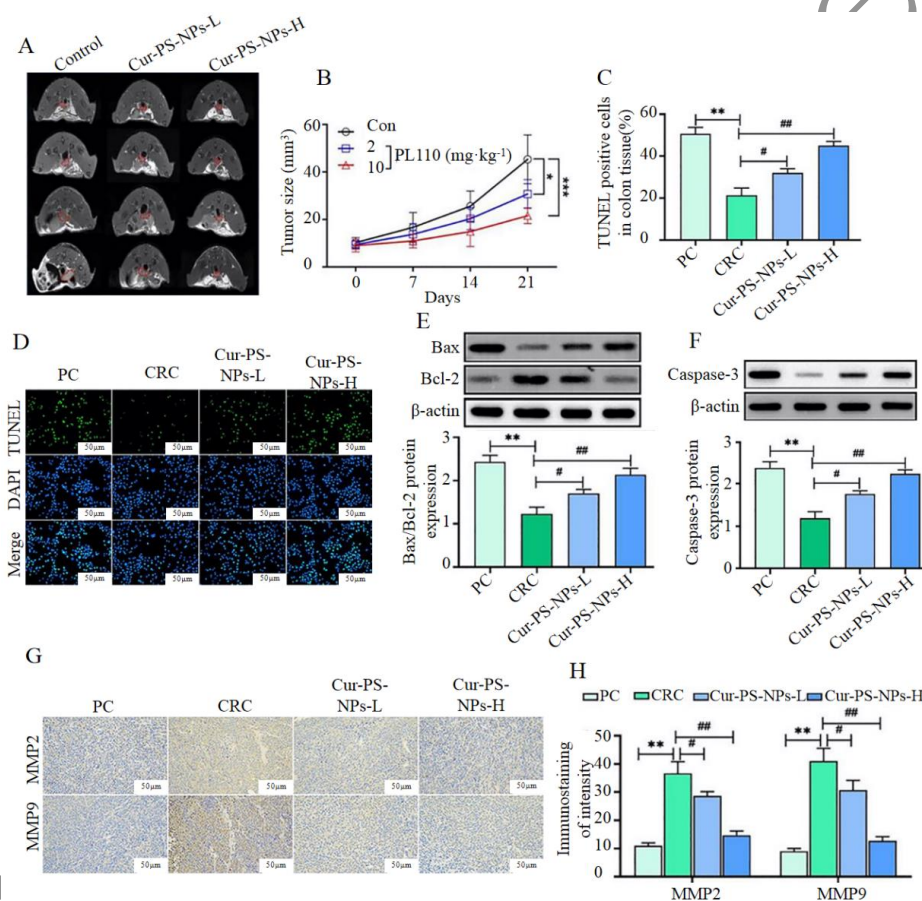


Fig. 3: The effect of Cur-PS-NPs on the biological behavior of tumor tissue in colorectal cancer mice. (A-B) MRC detection of tumor tissue size changes and statistical charts after 0, 7, 14, and 21 days after subcutaneous tumor transplantation in mice; (C-D) TUNEL detection of mouse tumor tissue apoptosis; (E-F) Western blot detection of mouse tumor tissue Expression of apoptosis-related proteins; (G-H) Immunohistochemical detection of expression of invasion and migration marker proteins in tumor tissues of mice. Compared with Con group, $*p < 0.05$, $***p < 0.001$; compared with PC group, $**p < 0.01$; compared with CRC group, $#p < 0.05$, $##p < 0.01$.

The polydispersity index is 0.15 ± 0.02 , indicating that the system is monodisperse. Furthermore, Cur-PS-NPs exhibited a negative surface charge, with a Zeta potential of -10.3 ± 2.1 mV (Fig. 1C). It indicates that the Cur-PS-NPs construction is successful.

Cur-PS-NPs can increase miR-186 expression in rectal cancer and inhibit EMT

Inject SW480 subcutaneously through the armpit of mice and observe the growth of subcutaneous colorectal cancer tumors in mice by MRC.

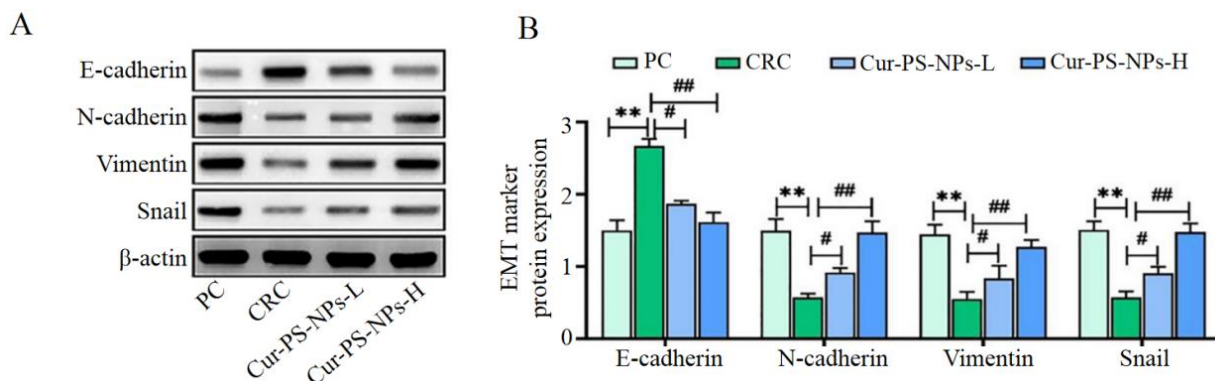


Fig. 4: (A) The effect of Cur-PS-NPs on the EMT of mouse tumor tissue; (B) Western blot detection of EMT marker protein expression levels and statistical charts in lung cancer cells in each group. Compared with PC group, $**p<0.01$; compared with CRC group, $#p<0.05$, $##p<0.01$.

The results showed that the mouse colorectal cancer tumor was growing and the colorectal cancer mouse model was successfully constructed, which can be used for subsequent experimental research (Fig. 2).

Further analysis showed that Cur-PS-NPs could inhibit the growth rate of CRC tumors (VS Control, $p<0.05$, Fig. 3A) and with the prolongation of intervention time, the growth trend of tumor diameter gradually decreased (VS Control, $p<0.05$, $p<0.001$, Fig. 3B). Cur-PS-NPs can also increase the apoptosis ability of cells in CRC tumor tissue and Bcl-2 expression as an anti-apoptotic protein was increased (VS CRC, $p<0.05$, $p<0.01$, Fig. 3C- 3F). Cur-PS-NPs can also reduce the expression of invasion and migration marker proteins MMP2 and MMP9 (VS CRC, $p<0.05$, $p<0.01$, Fig. 3G-3H). In the study of Cur-PS-NPs on the EMT of CRC mouse tumor tissue, it was found that E-cadherin expression was most reduced, while other markers of EMT were significantly enhanced (VS CRC, $p<0.05$, $p<0.01$, Fig. 4).

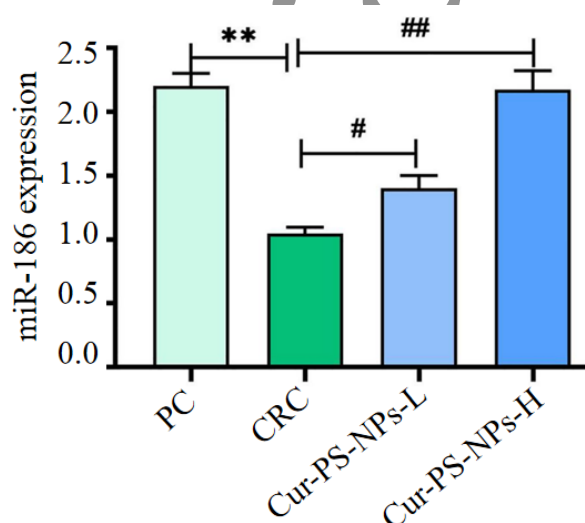


Fig. 5: Effect of Cur-PS-NPs on the expression of miR-186 in rectal cancer mice. qRT-PCR detection of miR-186 expression in mouse tumor tissues. Compared with PC

group, $**p<0.01$; compared with CRC group, $#p<0.05$, $##p<0.01$.

Subsequently, the expression level of miR-186 in tumor tissues was measured. It was found that Cur-PS-NPs increased miR-186 expression (VS CRC, $p<0.05$, $p<0.01$ Fig. 5).

Cur-PS-NPs can inhibit the expression of PI3K, AKT phosphorylation and TWIST in rectal cancer

Changes in the expression of the PI3K/AKT pathway and its downstream gene TWIST were then observed. Cur-PS-NPs reduced the level of phosphorylated proteins of PI3K and AKT (VS CRC, $p<0.05$, $p<0.01$ Figs. 6 and 7) and at the same time reduce the expression of TWIST.

Cur-PS-NPs can inhibit the EMT by increasing miR-186 expression and inhibiting PI3K/AKT signaling and down-regulating twist

In normal human colonic epithelial cells (NCM460), transfection with a miR-186 inhibitor significantly reduced miR-186 expression compared to the negative control (VS miR-186 NC, $p<0.001$, see Fig. 8).

Next, miR-186 interference fragments were transfected into rectal cancer cells. Transfection with the miR-186 inhibitor resulted in increased phosphorylation levels of PI3K and AKT (VS Cur-PS-NPs-H+miR-186 NC, $p<0.01$, Fig. 9). To determine whether Cur-PS-NPs co-regulate the expression of the downstream gene TWIST through miR-186 and the PI3K/AKT pathway, co-transfection experiments were performed in colorectal cancer cells. The results showed that after interference with miR-186 and inhibition of PI3K/AKT signaling, the expression levels of TWIST mRNA and protein were significantly increased (vs. Cur-PS-NPs-H + miR-186 inhibitor, $p<0.05$, Fig. 10). In order to clarify the effects of Cur-PS-NPs, miR-186 and PI3K/AKT signaling pathway on their biological behavior. The proliferation ability of cancer cells was restored after co-transfection (VS Cur-PS-NPs-H+miR-186 NC, $p<0.05$, Figs. 11A-11C).

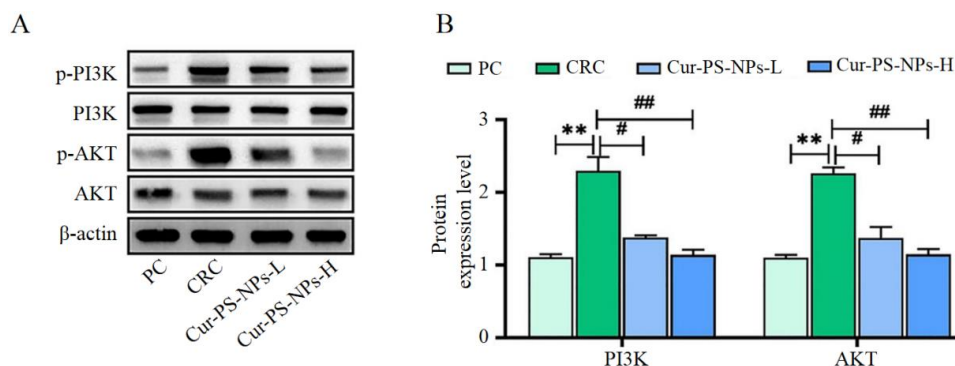


Fig. 6: (A) The effect of Cur-PS-NPs on the phosphorylation of PI3K and AKT in tumor tissues of rectal cancer mice; (B) Western blot detection of phosphorylation levels of PI3K and AKT and statistical charts. Compared with PC group, $**p < 0.01$; compared with CRC group, $\#p < 0.05$, $##p < 0.01$.

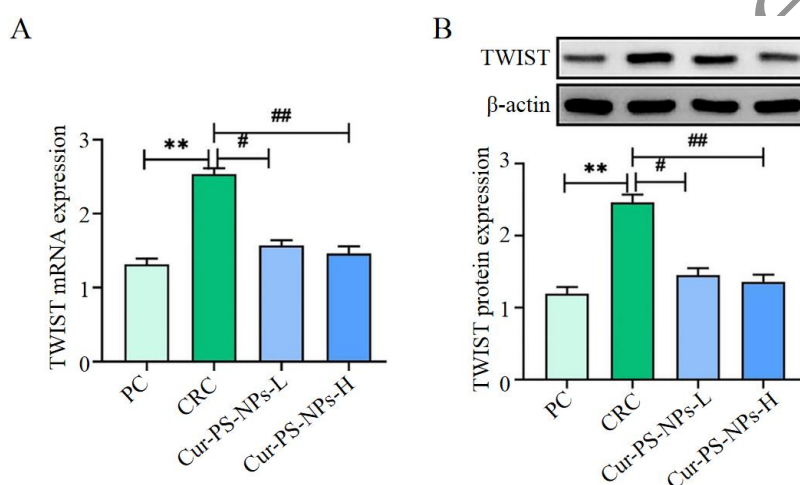


Fig. 7: The effect of Cur-PS-NPs on the expression of Twist in tumor tissues of rectal cancer mice. (A) qRT-PCR detection of Twist mRNA expression level; (B) Western blot detection of Twist protein expression changes. Compared with PC group, $**p < 0.01$; compared with CRC group, $\#p < 0.05$, $##p < 0.01$.

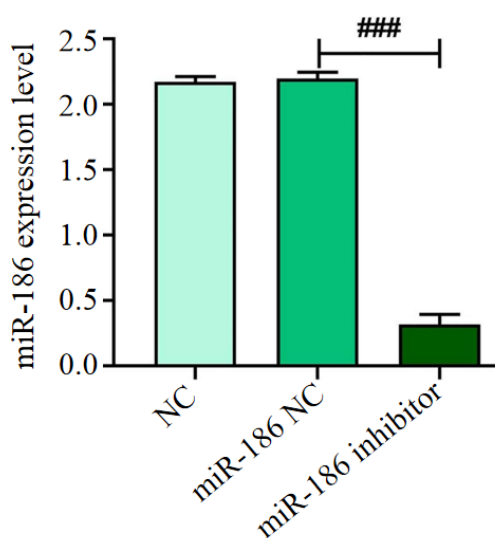


Fig. 8: Self-verification of interference miR-186. qRT-PCR detection of miR-186 expression in rectal cancer cells after miR-186 interference. $###p < 0.001$ compared to miR-186 NC group.

Apoptosis-related experiments found that the apoptosis ability of cancer cells decreased after co-transfection (VS Cur-PS-NPs-H+miR-186 NC, $p < 0.05$, Figs. 11D-11 G). In addition, the invasion and migration ability of cancer cells were enhanced after co-transfection. (Cur-PS-NPs-H+miR-186 NC, $p < 0.05$, Figs. 11H-11I).

E-cadherin expression was increased and the expression of other EMT marker proteins was decreased (Cur-PS-NPs-H+miR-186 NC, $p < 0.05$, Fig. 12).

DISCUSSION

CRC accounts for approximately 10% of new tumors worldwide (Hampel *et al.*, 2022). The rectal epithelium near the colon has a high content of microorganisms. (Uhlitz *et al.*, 2021; Jalandra *et al.*, 2023). EMT is involved in tumor cell migration (Huang *et al.*, 2022). Therefore, exploring the mechanism of EMT that effectively inhibits CRC is a hotspot of current research.

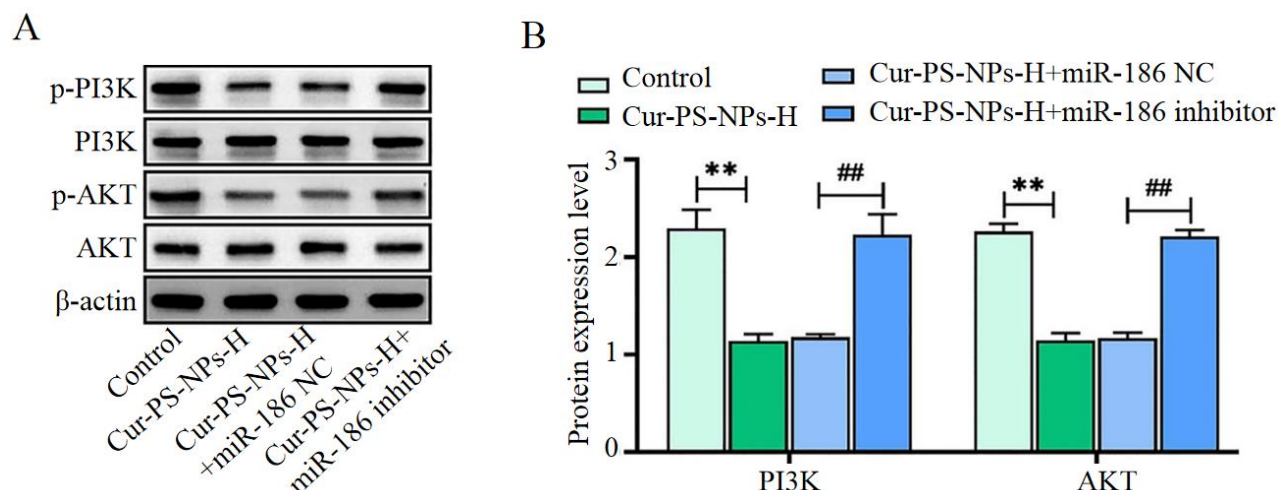


Fig. 9: Phosphorylation levels of PI3K and AKT after miR-186 interference. (A-B) Western blot detection of phosphorylation levels of PI3K and AKT and statistical charts. Compared with Control group, $^{**}p < 0.01$; compared with Cur-PS-NPs-H+miR-186 NC group, $^{##}p < 0.01$.

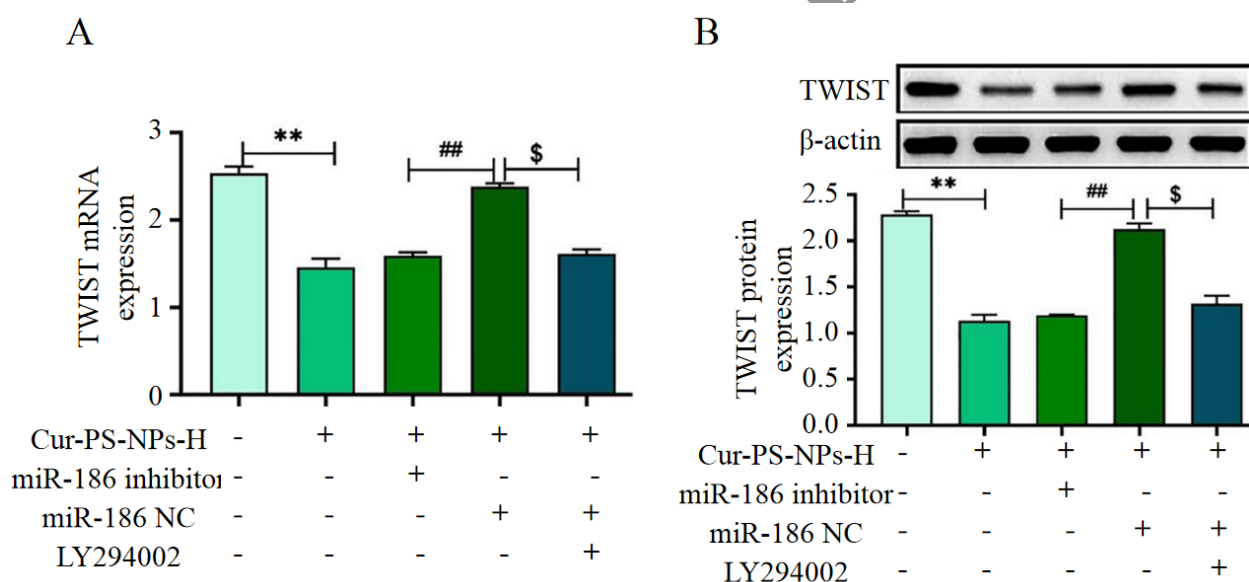


Fig. 10: The effect of miR-186 on the expression of Twist by regulating the PI3K/AKT pathway. qRT-PCR detection of Twist mRNA expression level; (B) Western blot detection of Twist protein expression changes. Compared with Control group, $^{**}p < 0.01$; compared with Cur-PS-NPs-H+miR-186 NC group, $^{##}p < 0.01$; compared with Cur-PS-NPs-H+miR-186 inhibitor group Ratio, $^{\$}p < 0.05$.

In this study Cur-PS-NPs were administered to CRC mice by intraperitoneal injection and found that Cur-PS-NPs could effectively slow down the growth of CRC tumors and inhibit the expression of invasion and migration marker proteins. At the same time, the apoptotic ability of tumor tissue was also detected and it was found that Cur-PS-NPs could also induce apoptosis of CRC tumor tissue.

This is consistent with the reported conclusion that Cur can prevent colon cancer growth (Li *et al.*, 2022). The superior efficacy observed with Cur-PS-NPs, however, likely stems from enhanced drug delivery mechanisms inherent to

nanoparticles. These include exploitation of the enhanced permeability and retention (EPR) effect for passive tumor accumulation, improved cellular internalization and protection of curcumin from rapid metabolism (Prasher *et al.*, 2021; Wilar *et al.*, 2024). These features collectively increase the intracellular bioavailability and retention of curcumin, enabling it to modulate the miR-186/PI3K/AKT/Twist axis more effectively and sustainably than the free compound. Cur is a phytochemical extracted from turmeric (Tiwari and Jain, 2020).

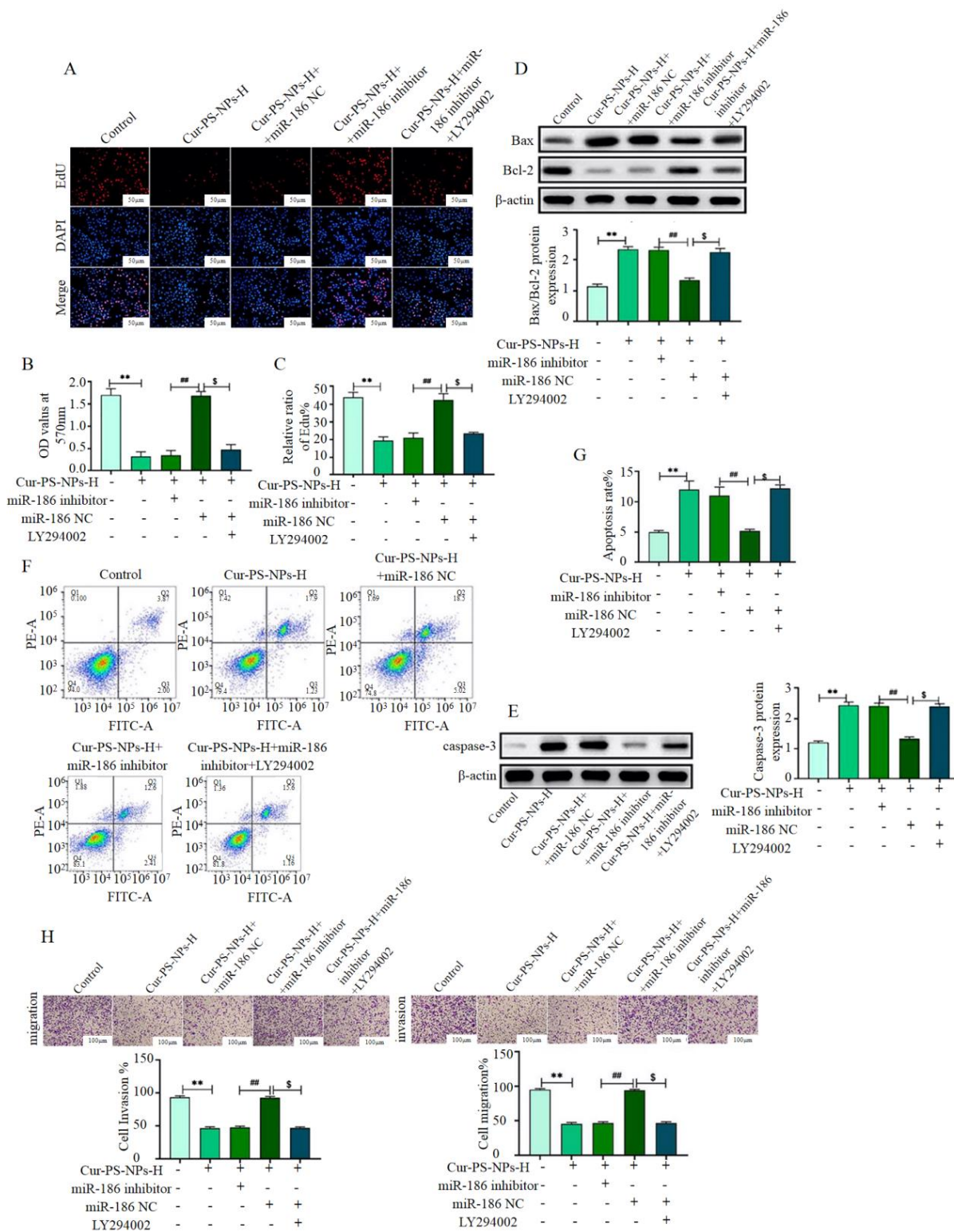


Fig. 11: (A) The effect of miR-186 on the biological behavior of colorectal cancer cells by regulating the PI3K/AKT pathway; (B) Edu detects the positive cells of rectal cancer cell proliferation; (C) MTT detects the proliferation of rectal cancer cells; (D-E) Western blot detects the expression of apoptotic proteins in rectal cancer cells; (F-G) Flow cytometry; (H-I) Transwell to examine the effect on migration and invasion of rectal cancer cells. Compared with Control group, $**p<0.01$; compared with Cur-PS-NPs-H+miR-186 NC group, $##p<0.01$; compared with Cur-PS-NPs-H+miR-186 inhibitor group ratio, $$p<0.05$.

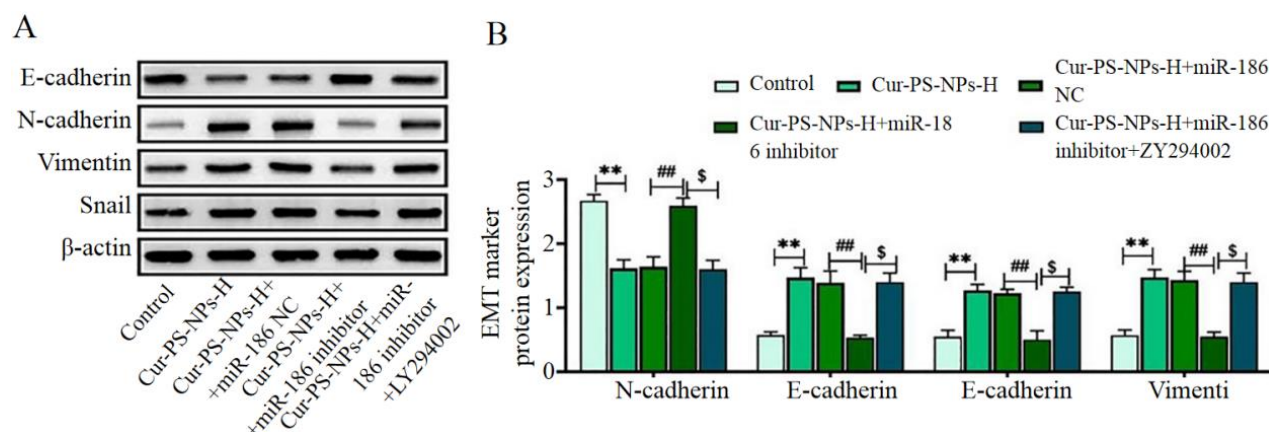


Fig. 12: The effect of miR-186 on the EMT of colorectal cancer cells by regulating the PI3K/AKT pathway. (A-B) Western blot detection of EMT marker protein changes in rectal cancer cells. Compared with Control group, $**p < 0.01$; compared with Cur-PS-NPs-H+miR-186 NC group, $##p < 0.01$; compared with Cur-PS-NPs-H+miR-186 inhibitor group ratio, $\$p < 0.05$.

Because it has a certain hydrophobicity, it can pass through the cell membrane and enter the endoplasmic reticulum with an anti-tumor effect (KariM *et al.*, 2025). The EMT of malignant tumors is closely related to the biological behavior of cells. Cur can promote protein synthesis in apoptosis, inhibit the invasion of epithelial cells and reduce the EMT of slowing tumors.

In the EMT transformation of pancreatic cancer cells treated by Cur, (Li *et al.*, 2020) found that Cur can affect EMT by inhibiting IL-6/ERK/NF- κ B axis and inhibit tumor-stromal transformation and metastasis of pancreatic cancer. This study found that Cur-PS-NPs can upregulate E-cadherin, while downregulating N-cadherin, Vimentin and Snail, indicating that Cur-PS-NPs can slow down the EMT of CRC mouse tumors and the experimental results of (Li *et al.*, 2020) are consistent. During EMT, the interaction of cells and extracellular matrix is remodeled, which can lead to the separation of epithelial cells and the underlying basement membrane, which leads to the increase of E-cadherin protein and activates a novel transcriptional program to promote EMT (Hashemi *et al.*, 2022). In the tumor environment, EMT can reduce the effect of therapeutic drugs and lead to drug resistance in the body. Therefore, it was found that Cur-PS-NPs could alleviate EMT by inhibiting tumor growth rate and increase the apoptosis level of CRC mice, so as to achieve the therapeutic effect of therapeutic drugs on colorectal cancer.

Further studies on the specific mechanism of Cur-PS-NPs inhibiting EMT in CRC revealed that it was also controlled by epigenetic changes. In this experiment, in CRC tumor tissues, miR-186 expression was significantly reduced, which was consistent with the findings of (Han *et al.*, 2020). Recent studies have shown increased miR-186 in CRC and closely related to CRC development. After CRC mice were treated with Cur-PS-NPs, the miR-186 level in the tumor tissue of CRC mice was restored and it could also

reduce Twist expression and PI3K and AKT phosphorylation were also reduced and suppressed. It has been reported in the literature that Twist is a key target gene controlling EMT. Lai found in liver cancer (Lai *et al.*, 2022) that Twist can be activated by the Fpg/Nei protein superfamily NEIL3, which promotes the development of EMT in liver cancer and produces resistance to therapeutic drugs. However, reducing the expression of Twist will reduce the EMT of tumor tissue (Jin *et al.*, 2021) found in breast cancer (Jin *et al.*, 2021) that FOXO3a can inhibit the EMT and migration of breast cancer by reducing the expression of Twist. It can be concluded that Cur-PS-NPs can regulate miR-186, EMT key target gene Twist and PI3K/AKT signaling pathway to affect EMT in CRC mice.

The precise regulatory nexus between miR-186, PI3K/AKT and Twist warrants further elaboration. While the data from this study confirm that Cur-PS-NPs upregulate miR-186, which in turn suppresses the phosphorylation of PI3K/AKT and the expression of Twist, the underlying molecular crosstalk remains to be fully mapped. miR-186 may directly target the 3' untranslated regions (3'UTRs) of key components within the PI3K/AKT pathway, such as PIK3CA (encoding the p110 α catalytic subunit of PI3K) or AKT itself, as suggested by bioinformatic predictions and studies in other cancers (Lu *et al.*, 2021; Chen and Zhang, 2022). The subsequent downregulation of Twist, a master transcriptional regulator of EMT, could be a direct consequence of attenuated PI3K/AKT signaling, as this pathway is a well-established upstream activator of Twist expression via inactivation of transcriptional repressors like FOXO proteins. Alternatively, or additionally, miR-186 might directly target Twist mRNA, forming a multi-level inhibitory network. This dual or multi-target potential of miR-186 underscores its potency as a therapeutic node and explains the profound anti-EMT effects observed with Cur-PS-NPs intervention.

To mechanistically dissect the causal relationships between miR-186, the PI3K/AKT pathway and Twist downstream of Cur-PS-NPs action, a series of *in-vitro* perturbation experiments were conducted. First, miR-186 expression was genetically interfered with in colonic epithelial cells. The interference of miR-186 could destroy the inhibitory effect of Cur-PS-NPs on PI3K/AKT signaling and promote phosphorylation of PI3K and AKT. Therefore, miR-186 might affect its activity by regulating the PI3K/AKT signal. Next, to explore whether the expression of EMT and its target gene Twist is affected by miR-186 and the PI3K/AKT signaling pathway, a PI3K inhibitor and miR-186 interference fragment were co-transfected into colorectal cancer cells, the results found that after co-transfection, Twist inhibited by Cur-PS-NPs was activated again, colorectal cells activities were further enhanced, apoptosis was gradually lost and the expression of EMT marker proteins also changed, in which E-cadherin was weakened and N-cadherin, Vimentin and Snail increased again. This suggests that changes in the expression of miR-186 and PI3K/AKT signaling pathways may affect the biological behavior and EMT of colorectal cancer cells and it can also regulate the expression of the Twist gene. This multi-level inhibitory network explains the profound anti-EMT effects observed. These findings thus position Cur-PS-NPs as not merely a delivery vehicle but as a sophisticated system that co-opts a key tumor-suppressive miRNA to disrupt a critical oncogenic axis in CRC.

Translating these findings to human CRC therapy presents both promise and challenges. The superior bioavailability and targeted efficacy of Cur-PS-NPs over free curcumin address a major historical limitation. The nano-formulation could potentially enhance drug accumulation in tumor tissue via the enhanced permeability and retention (EPR) effect while minimizing systemic side effects. Future preclinical developments must rigorously assess the pharmacokinetics, organ-specific biodistribution and potential immunogenicity of PS-NPs in human-relevant models. Furthermore, exploring combination therapies with standard chemotherapeutics like 5-fluorouracil or oxaliplatin could test for synergistic effects and overcome drug resistance mediated by EMT. Finally, identifying patient subgroups with low miR-186 expression or activated PI3K/AKT/Twist signaling could pave the way for personalized, biomarker-driven clinical trials of Cur-PS-NPs. Although the main purpose of this study is to explore the novel composite Cur-PS-NPs and its mechanism of action through the miR-186/PI3K/AKT/Twist axis, it must be acknowledged that the lack of control groups using free curcumin alone or PS-NPs alone constitutes a limitation in the experimental design. Including these control groups in future studies is essential for accurately dissecting the contribution of the nano-formulation. Specifically, this would help to clearly attribute the enhanced anti-tumor efficacy observed in this study to the improved bioavailability and targeted delivery provided by the PS-NP carrier, rather than solely to the

pharmacological activity of curcumin. Moreover, this would also help exclude any potential intrinsic effects of drug-unloaded PS-NPs on tumor growth or the EMT process.

CONCLUSION

In summary, this study can prove that Cur-PS-NPs can slow down the occurrence of EMT in CRC mice by regulating PI3K/AKT signaling. When miR-186 is disturbed, PI3K/AKT signaling will continue to increase. And after simultaneously interfering with miR-186 and inhibiting PI3K/AKT signaling, it will slow down EMT and TWIST expression. This provides a new idea for the research and development of EMT inhibitors for CRC. It should be noted that this proof-of-concept study utilized a mouse model and lacked control groups for individual components, specifically groups treated with free curcumin alone and blank PS-NPs alone. Future studies must prioritize the inclusion of these critical control groups. Additionally, it is necessary to employ more advanced models and conduct detailed pharmacokinetic research to advance this therapeutic approach towards clinical application.

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None.

Authors' contribution

Peinan Wen: Experimental design, data collection and analysis, manuscript writing; Jicai Chen: Experimental operations, research guidance, manuscript revision and review; Mingshu Lin: Material provision, data processing, technical support. All authors have read and agreed to the final manuscript.

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Data availability

The datasets generated and/or analyzed during the current study period can be obtained from the corresponding authors upon reasonable request.

Ethical approval

This study was approved by the Ethics Committee of Pingyang Hospital of Wenzhou Medical University (Approval No.: 2026004016). All animal procedures complied with the Guide for the Care and Use of Laboratory Animals and the Chinese Regulations for the Administration of Laboratory Animals. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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