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RESEARCH PAPER

Long noncoding RNA PCAT6 inhibits colon cancer cell apoptosis by regulating anti-apoptotic protein ARC expression via EZH2

Weimei Huang[a]*, Geng Su[b]*, Xiaoxian Huang[c]*, Angru Zou[d], Jingfang Wu[a], Yunchu Yang[a], Yaru Zhu[e], Shumei Liang[a], Deyu Li[f], Feng Ma[g], and Linlang Guo[a]

[a]Department of Pathology, Zhujiang Hospital, Southern Medical University, Guangzhou, China; [b]Department of Pathology, Minzu Hospital of Guangxi Zhuang Autonomous Region, Nanning, China; [c]Clinical Laboratory, Gushang Hospital of Guangxi Zhuang Autonomous Region, Nanning, China; [d]Department of Emergency, Minzu Hospital of Guangxi Zhuang Autonomous Region, Nanning, China; [e]Department of Cerebral Surgery, Zhujiang Hospital, Southern Medical University, Guangzhou, China; [f]Department of Oncology, Fujian Provincial Hospital, Fuzhou, China; [g]Department of Oncology, The First Affiliated Hospital of Hebei North University, Zhangjiakou, China

ABSTRACT

Prostate cancer-associated ncRNA transcript 6 (PCAT6) is a long intergenic noncoding RNA that is involved in the progression of prostate and lung cancer, acting as a potential diagnostic and prognostic biomarker in nonsmall cell lung cancer. However, little is known about PCAT6 expression and its clinical significance in colon cancer. Here, we aimed to investigate the clinical significance of PCAT6 in colon cancer and its underlying mechanism. The expression of PCAT6 was analyzed in colon cancer tissues using public databases, and a series of in vitro and in vivo experiments was performed to investigate the biological functions of PCAT6 in colon cancer cells and the underlying mechanisms. Our results demonstrated that PCAT6 was upregulated in colon cancer tissues compared with that in noncancerous tissues, correlating with poorer clinical stages and a worse survival status. In vitro and in vivo experiments illustrated PCAT6 promoted cell growth and inhibited cell apoptosis in colon cancer. Mechanistically, PCAT6 enhanced the coenrichment of EZH2 and H3K4me3 at the apoptosis repressor with caspase recruitment domain (ARC) genomic region, promoting the transcriptional activity of ARC. Our data highlighted that PCAT6 acts as a key activator of ARC expression by forming a complex with EZH2, inhibiting cell apoptosis and contributing to colon cancer progression. These findings elucidated that PCAT6 may be a novel prognostic predictor and therapeutic target of colon cancer.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers, the third and fourth leading causes of cancer-related mortality worldwide in women and men, respectively [1-3]. Approximately two-thirds of CRC cases are colon cancers. Thanks to screening, a healthy lifestyle and substantial advances in cancer treatment, the mortality rates of patients with advanced colon cancer have decreased over the last decade. However, the 5-year overall survival rate of colon cancer remains less than 65% [4]. Because of the lack of early signs, rapid progression, and resistance of chemotherapy, the need to identify early diagnostic biomarkers and potential therapeutic targets to improve colon cancer treatment is urgent.

Long noncoding RNAs (lncRNAs) are RNA transcripts that are longer than 200 nucleotides and lack an open reading frame of significant length [5]. A sufficient amount of evidence has suggested that lncRNAs constitute an important component of tumor biology via different molecular mechanisms, including interaction with DNA, RNA, and proteins [6,7]. Thus far, a small fraction of lncRNAs was shown to play a critical role in the physiological and pathological processes of cancer. For example, the lncRNA LINC-PINT inhibits tumor cell invasion through regulating the availability of free PRC2 at the proximity of coregulated genomic loci [8]. The lncRNA MetaLnc9 facilitates lung cancer metastasis by regulating the AKT/mTOR pathway [9]. The lncRNA FAL1, which is overexpressed in epithelial tumors, is associated with the epigenetic repressor BMI1 and regulates the transcription of several genes, promoting tumor growth [10]. MALAT1 is involved in renal cell carcinoma progression by interacting with EZH2 [11]. The

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lncRNA EGFR-AS1 mediates epidermal growth factor receptor addiction and modulates the drug resistance of squamous cell carcinoma [11]. However, the potential regulatory roles and functions of lncRNAs in colon cancer progression remain largely unknown.

An integral part of the carcinogenic process in tumors is the imbalance between cell proliferation and cell apoptosis, making tumors more challenging to cure [12]. In many cancers, hyperactivation mutations or overexpression of anti-apoptotic proteins are frequently observed [13]. ARC is an anti-apoptotic protein encoded by nucleolar protein 3 (NOL3) and has been reported to be increased in primary human epithelial cancers, including breast, colon, cervical, and ovarian cancer, compared with the corresponding normal tissues, conferring cancer progression via inhibiting the activity of caspase-8 and caspase-2 as well as antagonizing both the extrinsic (death receptor) and intrinsic (mitochondrial/ER) pathways [14,15]. However, the functions of ARC in colon cancer remain obscure.

In our study, by analyzing the lncRNA expression profile in colon cancer tissues and paired adjacent noncancerous tissues using public databases, we identified PCAT6, a 764-bp-long intergenic lncRNA located at 1q32, that is frequently elevated in colon cancer tissues and is correlated with the clinical stage and overall survival of patients. The biological role of PCAT6 in colon cancer cells was assessed genetically both in vitro and in vivo. We demonstrated that PCAT6 activates ARC expression via increasing EZH2 and H3K4me3 co-occupancy at ARC genomic loci, leading to the inhibition of cell apoptosis in colon cancer. Finally, we showed that silencing ARC expression could mitigate PCAT6-induced anti-apoptosis behavior in vitro. Our expression, functional, and mechanistic data illustrated that PCAT6 is highly associated with colon cancer malignancy and might be a robust biomarker for prognosis and a potential therapeutic target in colon cancer.

Materials and methods

Cells and reagents

HCT116, RKO, COLO320HSR, SW480, and SW620 cell lines were purchased from the American Type Culture Collection (ATCC, USA) and were maintained according to the manufacturer's instructions. The NCM460 cell line was obtained from the Cellular Institute of Chinese Academy of Science (Shanghai, China) and was maintained in DMEM medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/mL of penicillin and 100 mg/mL of streptomycin (Solarbio, English) and was cultured in a humidified 5% CO_2 incubator at 37°C. The six cell lines used in this study were not contaminated by mycoplasma.

GAPDH (catalog AP0063; 1:5,000) antibody was acquired from Bioworld Technology. ARC (NOL3; catalog 10846-2-AP; 1:500) antibody was acquired from Proteintech Group. H3K4me3 (catalog 9751; Chromatin IP: 10 μl of antibody per IP), cleaved Caspase3 (catalog 9664; 1:1000), EZH2 (catalog 5246; western blotting: 1:1,000; chromatin IP: 5 μl of antibody per IP) and rabbit IgG (catalog 3900) antibodies were acquired from Cell Signaling Technology. HRP-conjugated secondary antibodies (1:1,000) were acquired from Santa Cruz Biotechnology.

Other reagents used in this study were as follows: AnnexinV APC (catalog V35113; Thermo Fisher Scientific), PI (catalog R37108; Thermo Fisher Scientific), Lipofectamine™ 3000 Transfection Reagent (catalog L3000015; Thermo Fisher Scientific), and Cell Counting Kit-8 (catalog CK04; Dojindo Laboratories).

Expression data set

Online-available datasets were downloaded from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) and The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/). R language and Bioconductor were used for data quality control, expression normalization, calculation, and annotation [16]. Five public cohorts were used in this study: GSE21510 and GSE32323 showed microarray expression profiling containing normal colonic epithelium tissues and colon cancer tissues. GSE36070 showed analysis of the EZH2-interacting RNAs in HCT116 cells using an in vivo cross-linking and immunoprecipitation strategy (iCLIP). GSE85766 showed H3K4me3, H3K27me3, and H3K79me3 CHIP-sequencing data performed in colon cancer cell lines. TCGA-COAD files were generated based on the MapSplice algorithm for alignment against the hg19

reference genome using default parameters [17]. Student's t-test or analysis of variance (ANOVA) was used to detect the significant difference between two or more groups.

RNA extraction and qPCR assays

Total RNA was extracted from xenograft tumors or cultured cells using TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions, and then the total RNA concentration was determined using Nanodrop 2000 (Thermo). Total RNA (1,000 ng per sample) was reverse transcribed in a final volume of 20 μl using random primers under standard conditions for the FastKing RT Kit (catalog KR116; Tiangen biotech, Beijing, China). PCR analysis was performed using SYBR Green Talent qPCR PreMix (catalog FP209; Tiangen biotech, Beijing, China), and amplified PCR products were quantified and normalized using the housekeeping gene GAPDH as a control. Quantitative PCR (qPCR) and data collection were carried out using a Bio-Rad CFX connect real-time PCR system (Bio-Rad Laboratories, USA). The primers for the genes of interest were synthesized by Sangon Biotech (Shanghai, China). The sequences for the gene-specific primers used in this study were as follows:

PCAT6-F: 5′- TCCTCATTCGGTCCATCCAA CTCC-3′;
PCAT6-R: 5′-GAAGCACGAGCAAGGCAGAG AC-3′;
ARC-F: 5′-GTCAGAGACTATCGACCGC-3′;
ARC-R: 5′-ATCCAATGCCTCGTACTCTG-3′;
EZH2-F: 5′-AAATCAGAGTACATGCGACTG A-3′;
EZH2-R: 5′-GTATCCTTCGCTGTTTCCATTC -3′;
GAPDH-F: 5′-GGAGCGAGATCCCTCCAAAA T-3′;
GAPDH-R: 5′-GGCTGTTGTCATACTTCTCA TGG-3′.

Vector construction, short hairpin RNA (shRNA) synthesis and transfection

shRNAs specifically against EZH2, ARC, and non-specific shRNA-control were designed and then were cloned into the pSiCoR shRNA vector by GenePharma (Suzhou, China). shRNAs were introduced into HCT116 cells or RKO cells using the Lipofectamine™ 3000 Transfection Reagent following the manufacturer's instructions. The efficiency of transfection was determined by the qRT-PCR assay. After culturing for 24 or 48 h, the transfected cells were harvested for in vitro studies. The sequences of shRNAs used in this study were as follows:

shEZH2 #1: 5′-GAGGGAAAGUGUAUGAUAA TT-3′;
shEZH2 #2: 5′-GCUCCUCUAACCAUGUUUA TT-3′;
shARC #1: 5′-GAGUACGAGGCAUUGGAUGC ATT-3′;
shARC #2: 5′-GAGGCCUCUAAAGAGGCUGA ATT-3′.

Lentivirus production and construction of stable cell lines

Full-length PCAT6 cDNA was commercially synthesized and then was cloned and inserted into the lentiviral expression vector pLV5/EF-1a/GFP/Puro (GenePharma, Suzhou, China). To generate lentiviral particles, HEK-293T cells were cotransfected with the resulting vector and packaging plasmids (GenePharma) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's guidelines. Infectious lentivirus was harvested at 48 h posttransfection, was filtered through filters, and then was concentrated using Lenti-X Concentrator (Clontech). The lentiviral particles were collected to infect SW480 and SW620 cells. Next, the lentivirus-infected cells were selected with the antibiotic puromycin. The recombinant lentivirus cells were designated as SW480-LV-PCAT6 and SW620-LV-PCAT 6. We used the empty lentiviral expression vector as a negative control and designated those cells as SW480-LV-empty and SW620-LV-empty.

Two pairs of cDNA oligonucleotides to silence PCAT6 expression were commercially designed and synthesized. After annealing, the double-strand oligonucleotides were inserted into the lentiviral vector pLV3/H1/GFP/Puro (GenePharma). The process of obtaining lentiviral particles was performed as described above. The lentiviral

particles were used to infect HCT116 and RKO cells, and then these cells were selected with the antibiotic puromycin. Stable PCAT6 knockdown cells were designated as LV-shPCAT6 #1 and LV-shPCAT6 #2. We used a scrambled shRNA as a negative control and designated it as LV-shCtrl.

Proliferation and colony formation assays

Cell proliferation was monitored using Cell Counting Kit-8 (Dojindo Laboratories): 2×10^3 cancer cells were seeded in 100 µl of complete growth medium into 96-well plates. The plates were incubated for 24 h, and the cell viability was assessed every day for five days according to the manufacturer's protocol. The cell proliferation curves were plotted using the absorbance at each time point by GraphPad Prism software.

For colony formation assays, 300 cancer cells were seeded into six-well plates, were maintained in basal medium containing 10% FBS and were allowed to grow for 15 days. The cells were fixed in 4% paraformaldehyde (Solarbio, English) and were stained with 0.5% crystal violet (Sigma Aldrich, St. Louis, MO, USA) on day 15. The number of stained colonies was counted.

Flow cytometric analysis

Cells for apoptosis or dead analysis were stained with Annexin V or propidium iodide (PI) using the Apoptosis Detection Kit APC (eBioscience, Thermo Fisher Scientific) following the manufacturer's protocol and were analyzed by FACScan (BD Biosciences). Apoptotic cells were defined as cells with Annexin V-positive staining.

For cell cycle analysis, the cells were fixed with 70% ice-cold ethanol for 2 hours and were stained with PI in the presence of RNase A (Qiagen) and then were analyzed by FACScan. The percentage of the cells in G0–G1, S, and G2–M phase were counted and compared. All samples were assayed in triplicate.

Western blot assay

Frozen cell pellets were lysed in RIPA buffer (Beyotime, China) supplemented with protease and phosphatase inhibitor cocktail (CWBIO, Beijing,

China). The protein concentration was quantified using a BCA protein assay kit (CWBIO, Beijing, China). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore Sigma, USA). All primary antibodies were incubated overnight at 4°C with membranes in primary antibody dilution buffer (Beyotime, China), while secondary antibodies were incubated at room temperature with agitation for 1 h in primary blocking buffer. The immune complex was detected using the ECL kit (Millipore Sigma, USA). The amounts of protein relative to the loading control were quantified by ImageJ software.

Tumor xenograft assay

Research involving animals were performed in compliance with the policies of the animal ethics committee of Southern Medical University of China. Female Balb/c nude mice (4–5 weeks of age) were used for the experiments. The mice were injected subcutaneously into the right flank with 1×10^6 cancer cells per site. The tumor volumes were measured every 3 to 6 days and were calculated using the following formula: $((\text{major axis}) \times (\text{minor axis})^2)/2$. The mice were sacrificed at the endpoint. The tumors were removed and weighed individually. The tumors were used for subsequent research.

RNA immunoprecipitation

HCT116 cells were cultured in 75-cm^3 cell culture flasks, and the RIP assay was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (catalog 17–701, Millipore) following the manufacturer's protocol. Rabbit lgG was used as the negative control. Coimmunoprecipitated RNA was extracted and analyzed by qRT-PCR. The primer sequences of PCAT6 for qRT-PCR were described above. The human EZH2 antibody was purchased from Cell Signaling Technology.

Chromatin immunoprecipitation (CHiP)–qPCR

Chromatin IP was performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit (catalog 17-295, Millipore) as directed by the manufacturer. Briefly, HCT116-LV-shPCAT6, HCT116-LV-shCtrl,

SW620-LV-PCAT6, and SW620-LV-empty cells were used to perform immunoprecipitation. Anti-H3K4me3, anti-EZH2, and Rabbit lgG were immunoprecipitated with protein-DNA complexes. Next, the immunoprecipitated DNA was purified and used for qPCR analysis. The ChIP-qPCR primer sequences used in this study were as follows:

> ARC-Chip-primer-F: 5′-GTCAGAGACTATCG ACCGCGA-3′;
> ARC-Chip-primer-R: 5′-GCATCCAATGCCTC GTACTT-3′.

The resulting signals were normalized for primer efficiency by carrying out qPCR for the ARC primer pair using input DNA (pooled unprecipitated DNA from cells).

Statistical analysis

Statistical significance, including two-tailed unpaired Student's t-test, one-way ANOVA, univariate analysis and the Cox proportional hazards model, was analyzed by SPSS22.0. Pearson and Spearman's correlation were performed using GraphPad Prism software. The data were represented as means ± SD (n > 10) or means ± SEM (n ≤ 10). Gene expression and patient clinical data extraction were analyzed using R language and Bioconductor. All experiments in this study were repeated at least three times independently. A P value less than 0.05 was considered to indicate statistical significance. Throughout this study, *: p < 0.05; **: p < 0.01; ***: p < 0.001.

Results

PCAT6 is highly expressed in colon cancer and is associated with a poor patient prognosis

To identify colon cancer-relevant lncRNAs, we analyzed the differential genes between colon cancer tissues and adjacent noncancerous tissues of three online-available datasets downloaded from GEO (https://www.ncbi.nlm.nih.gov/geo/, GSE21510 and GSE32323) and TCGA (https://cancergenome.nih. gov/, COAD). Among the significantly deregulated transcripts, we identified a novel lncRNA PCAT6 that was significantly upregulated in colon cancer tissues (Figure 1(a)). To exclude the influence of differences between individuals, we further analyzed

the expression of PCAT6 in 58 paired colon cancer tissues in these datasets and found that it was significantly upregulated in 53 of the colon cancer tissues compared with their adjacent normal tissues (P < 0.05) (Figure 1(b)). Interestingly, statistical analysis of the 439 RNA-seq transcriptome profiles of the TCGA-COAD dataset revealed PCAT6 to be significantly upregulated from the early clinical stages (I and II) to advanced stages (III and IV) (Figure 1(c)). Importantly, high levels of PCAT6 were remarkably associated with a worse overall patient survival (Figure 1(d)). The rates of high expression of PCAT6 in colon cancer with respect to clinicopathologic features are detailed in Table 1. The results demonstrated that high expression of PCAT6 had a significant correlation with the tumor subtype, N classification, metastasis, poorer clinical stage and a worse survival status of patients. However, no significant differences were observed with respect to gender (female and male) and age (≤66 years and >66 years) in our analysis. Spearman's analysis of the correlation between PCAT6 and clinicopathological features showed that the expression of PCAT6 was significantly correlated with the clinical stage, N classification, metastasis, tumor subtype and vital status (Table 2). Furthermore, univariate analysis showed that the following factors were significantly associated with a worse overall survival of colon cancer patients: age, stage, and PCAT6 expression. Additionally, multivariate analysis using the Cox proportional hazards model indicated that age, stage, and PCAT6 expression were associated with overall survival (Table 3). These results suggested that PCAT6 is highly expressed in colon cancer and is related to the clinical severity and prognosis.

PCAT6 promotes the cell growth and inhibits the cell apoptosis of colon cancer in vitro

We further investigated the possible role of PCAT6 in colon cancer progression. PCAT6 expression was determined in five colon cancer-derived cell lines by qRT-PCR (Figure 2(a)). All the colon cancer-derived cell lines showed higher PCAT6 levels than the non-tumoral colon epithelial cell line NCM460. Next, we successfully established four stable PCAT6-silenced cell lines (HCT116-LV-shPCAT6 #1, HCT116-LV-shPCAT6 #2, RKO-LV-shPCAT6 #1, and RKO-LV-
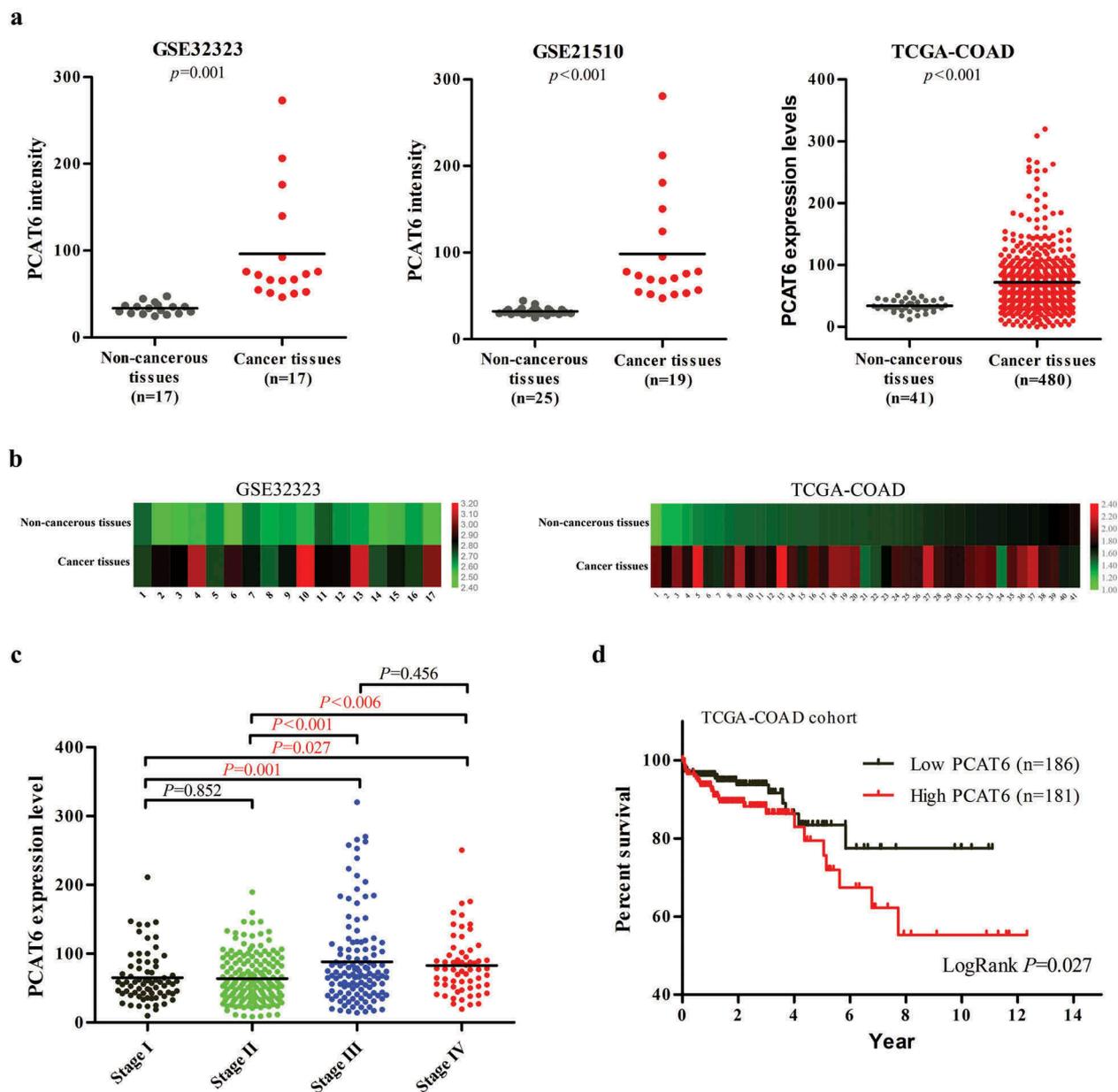
Figure 1. PCAT6 is upregulated in colon cancer and its clinical features. (a) PCAT6 was highly expressed in colon cancer tissues, data were downloaded from the GEO database and TCGA database. (b) PCAT6 expression was markedly increased in 53 paired colon cancer tissues (cancer) compared with their adjacent normal tissues (noncancerous) in GSE32323 and TCGA-COAD profiles. (c d) The expression of PCAT6 was related to the clinical stage (c) and overall survival (d) of colon cancer patients according to the TCGA-COAD cohort.

shPCAT6 #2; LV-shCtrl represents the scrambled shRNA control) via lentiviral infection of HCT116 and RKO cells (Figure 2(b)). Our results showed that silencing PCAT6 decreased cell growth compared with scrambled shRNA control cells (Figure 2(c)). Additionally, colony formation assay also revealed silencing PCAT6 inhibited cell growth (Figure 2(d)). We used flow cytometry to measure the cell cycle distribution and observed no significant differences in cell cycle arrest after silencing PCAT6 (data not

shown). We supposed that PCAT6 might affect cell survival via reducing cell apoptosis. To verify our hypothesis, the cells were analyzed by flow cytometry and were confirmed by western blotting for the apoptosis protein marker cleaved caspase 3. As expected, the results showed that the loss of PCAT6 resulted in increased cell apoptosis and induced cleaved caspase 3 activation (Figure 2(e,f)).

Because shRNA depletion studies may demonstrate off-target effects, we subsequently

Table 1. Correlation between PCAT6 expression and the clinicopathologic characteristics of colon cancer patients.

characteristics PCAT6	PCAT6 Low expression	High expression	p value
Age (years)			
≤66	116	73	0.706
>66	137	93	
Gender			
Female	122	73	0.394
Male	133	93	
Clinical stage			
I	52	19	<0.001
II	109	53	
III	64	55	
IV	24	35	
T classification			
T1	8	3	0.176
T2	50	22	
T3	167	116	
T4	27	25	
N classification			
N0	169	78	<0.001
N1	49	49	
N2	35	39	
Metastasis			
No	204	103	<0.001
Yes	47	58	
Subtype			
COAD_CIN	118	103	0.001
COAD_GS	34	15	
COAD_MSI	46	12	
COAD_POLE	5	1	
Status			
Alive	208	114	0.001
Dead	45	52	

Table 2. Spearman analysis of the correlation between PCAT6 and clinicopathological characteristics.

Variables	PCAT6 expression level Spearman Correlation	p value
Age	0.018	0.707
Gender	0.042	0.395
Clinical stage	0.216	<0.001
T classification	0.108	0.028
N classification	0.195	<0.001
Metastasis	0.194	<0.001
Subtype	−0.22	<0.001
Status	0.157	0.001

investigated the effect of PCAT6 overexpression in colon cancer cells. We upregulated PCAT6 expression in SW480 and SW620 cells by infecting

lentiviral vectors (Figure 3(a)). We observed a slight increase in cell growth and decrease in cell apoptosis in vitro (Figure 3(b-e)).

Taken together, our results suggested that PCAT6 promotes colon cancer cell survival in vitro, and the biological processes affected by PCAT6 are related to colon cancer cell apoptosis.

PCAT6 contributes to the tumorigenesis of colon cancer cells in vivo

To further elucidate the effect of PCAT6 on tumorigenesis in vivo, we employed a xenograft mouse model to query whether PCAT6 plays an important role in tumor growth. Colon cancer cells were subcutaneously injected into the flanks of nude mice. The growth of tumors was measured every 3 to 6 days for 5 weeks, and xenograft tumors were removed for final analysis. Our results showed that the tumor volumes from xenografts with reduced PCAT6 expression were significantly decreased compared with cells with higher PCAT6 expression (Figure 4(a,b)). Similarly, there was a significant delay in the onset of primary tumor formation when HCT116 cells with PCAT6 knockdown were injected into flanks compared with cells with a negative control vector (Figure 4(c)). Persistent knockdown of PCAT6 in these tumors was confirmed by the qRT-PCR assay (Figure 4(d)).

On the other hand, we determined whether exogenously elevated PCAT6 expression in colon cancer cells could effectively promote the growth of tumors in vivo. We generated stable PCAT6 overexpression cell lines via the lentiviral infection of SW620 cells (SW620-LV-PCAT6); control cells were transduced with empty lentivirus (SW620-LV-empty). Our results demonstrated that the tumor volume and tumor weight were dramatically higher in the SW620-PCAT6 group than in the SW620-empty

Table 3. Univariate and multivariate analyzes of various prognostic parameters in patients with colon cancer Cox-regression analysis.

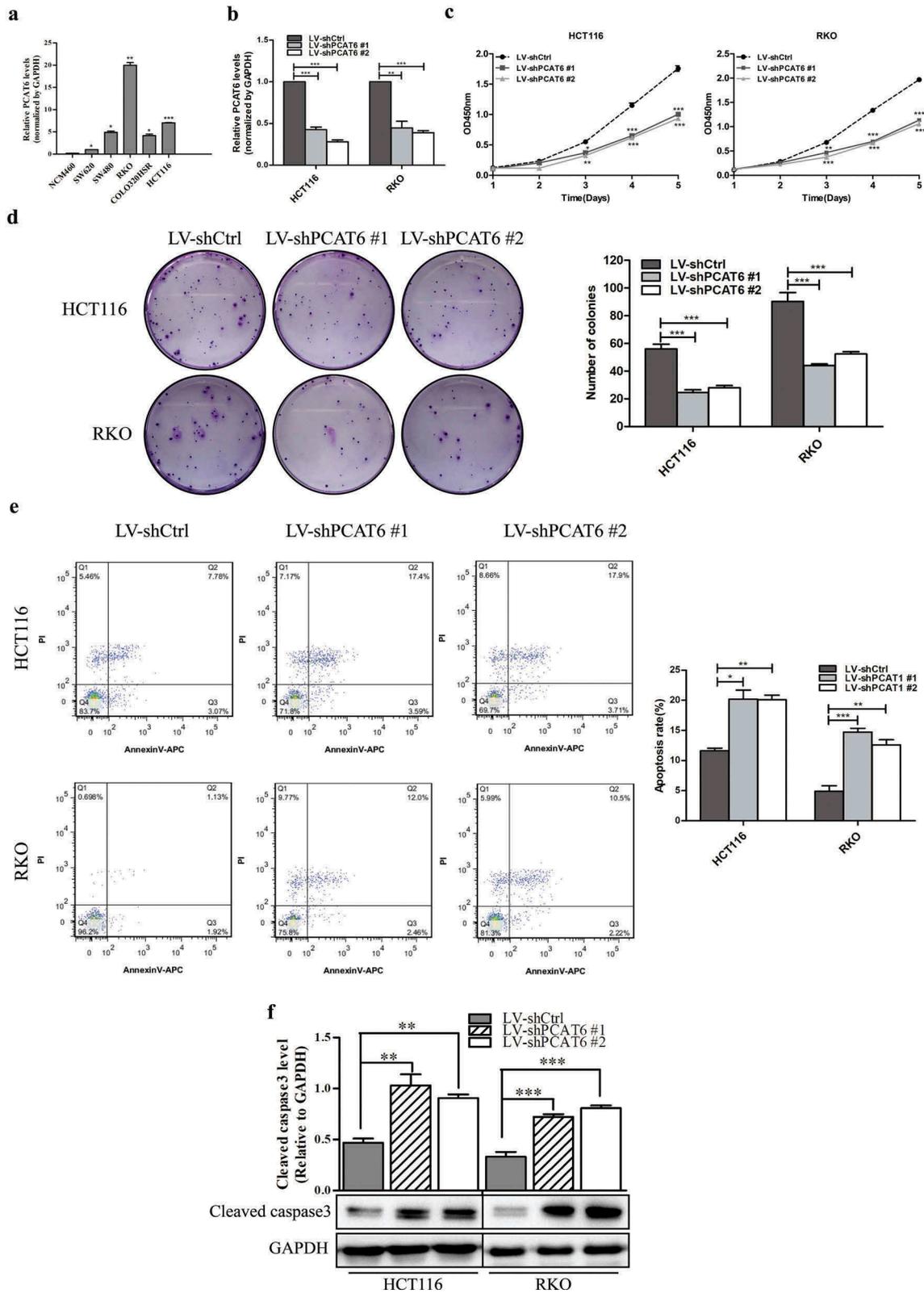|	Univariate analysis		Multivariate analysis		
	p	Hazard Ratio	p	Hazard Ratio	95% confidence interval
Age	0.019	2.415	0.001	4.603e+06	766.23–2.765e+10
Gender	0.661	0.860	0.922	1.06	0.35–3.19
Clinical stage	<0.0011	5.913	<0.001	35.95	10.33–125.07
PCAT6	0.002	1.457	0.020	4.03	1.25–13.06

Figure 2. PCAT6 affects the cell growth and cell apoptosis of colon cancer. (a) PCAT6 was evaluated in colon cancer cell lines compared with the normal colon epithelial cell line NCM460 analyzed by the qRT-PCR assay. (b) PCAT6 was effectively knocked down by shRNAs. (c d) Knocked down PCAT6 inhibited colon cancer cell growth, as determined by the CCK8 assay (c) and cell colony formation assay (d). (e) Silencing PCAT6 increased cell apoptosis. (Left: Representative FACS profiles are shown, among which the cell population in the quadrant of Annexin V± PI represents apoptotic cells. Right: The percentage of apoptotic cells is shown in histogram form). (f) Western blot analysis revealed an increase in the apoptosis marker cleaved caspase 3 in HCT116 or RKO cells after stable knock down of PCAT6.
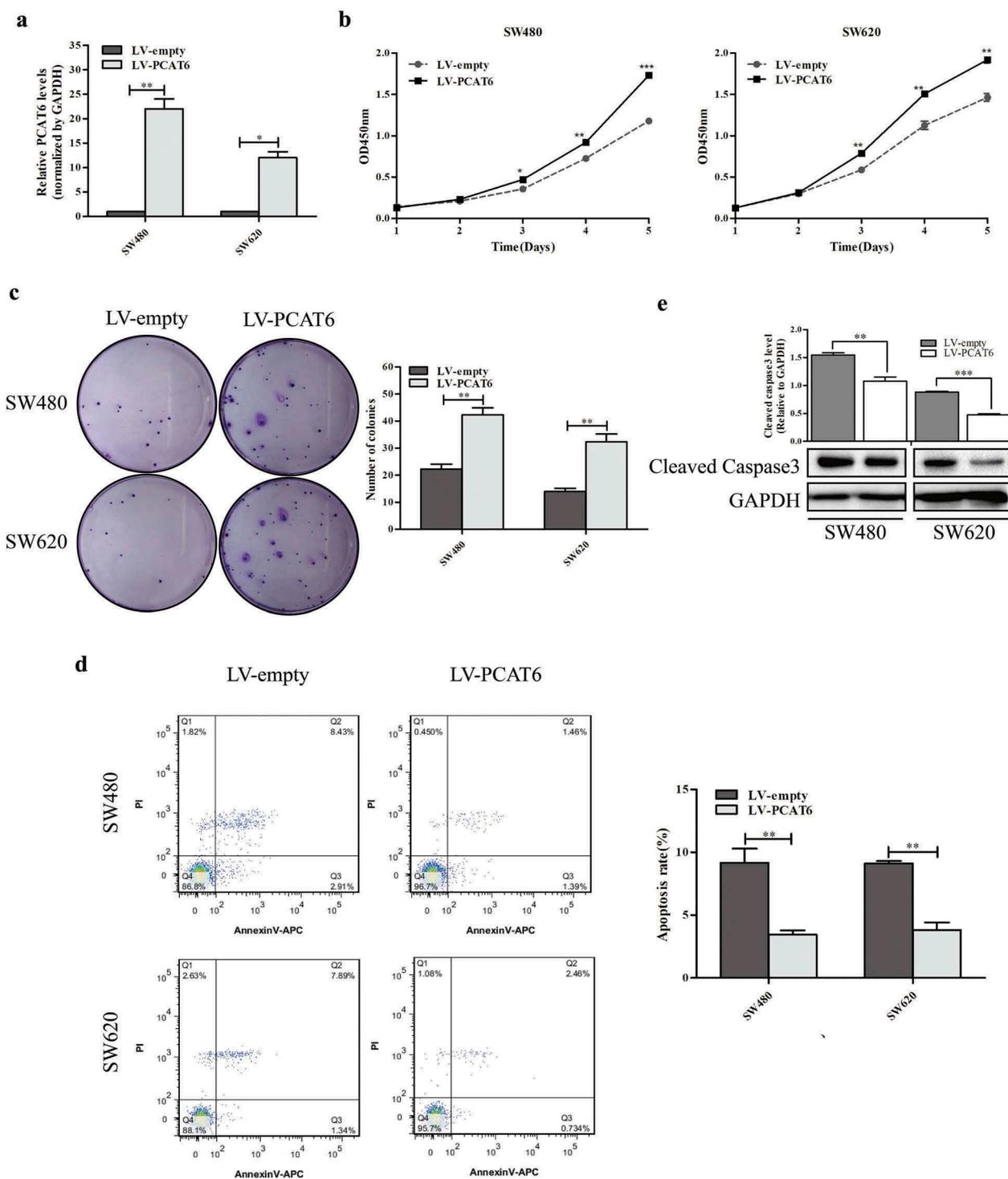
Figure 3. PCAT6 affects the cell growth and cell apoptosis of colon cancer. (a) Successfully established stable high expression of PCAT6 in SW480 and SW620 cells via lentiviral vector transfection. (b c) Cell proliferation assay (b) and cell colony formation assay (c) for SW480 and SW620 cells that stably overexpressed PCAT6 or negative controls. (d) Enforcing PCAT6 expression decreased cell apoptosis in colon cancer cells, as measured by flow cytometry. (e) Colon cancer cell lysates were immunoblotted for cleaved caspase 3.

control group (Figure 4(e,f)). Similarly, overexpression of PCAT6 in cells showed a significantly faster tumor growth (Figure 4(g)), suggesting that PCAT6 substantially promoted cancer cell tumorigenesis in vivo. Next, the qRT-PCR assay was used to evaluate the levels of PCAT6 in tumors (Figure 4(h)).
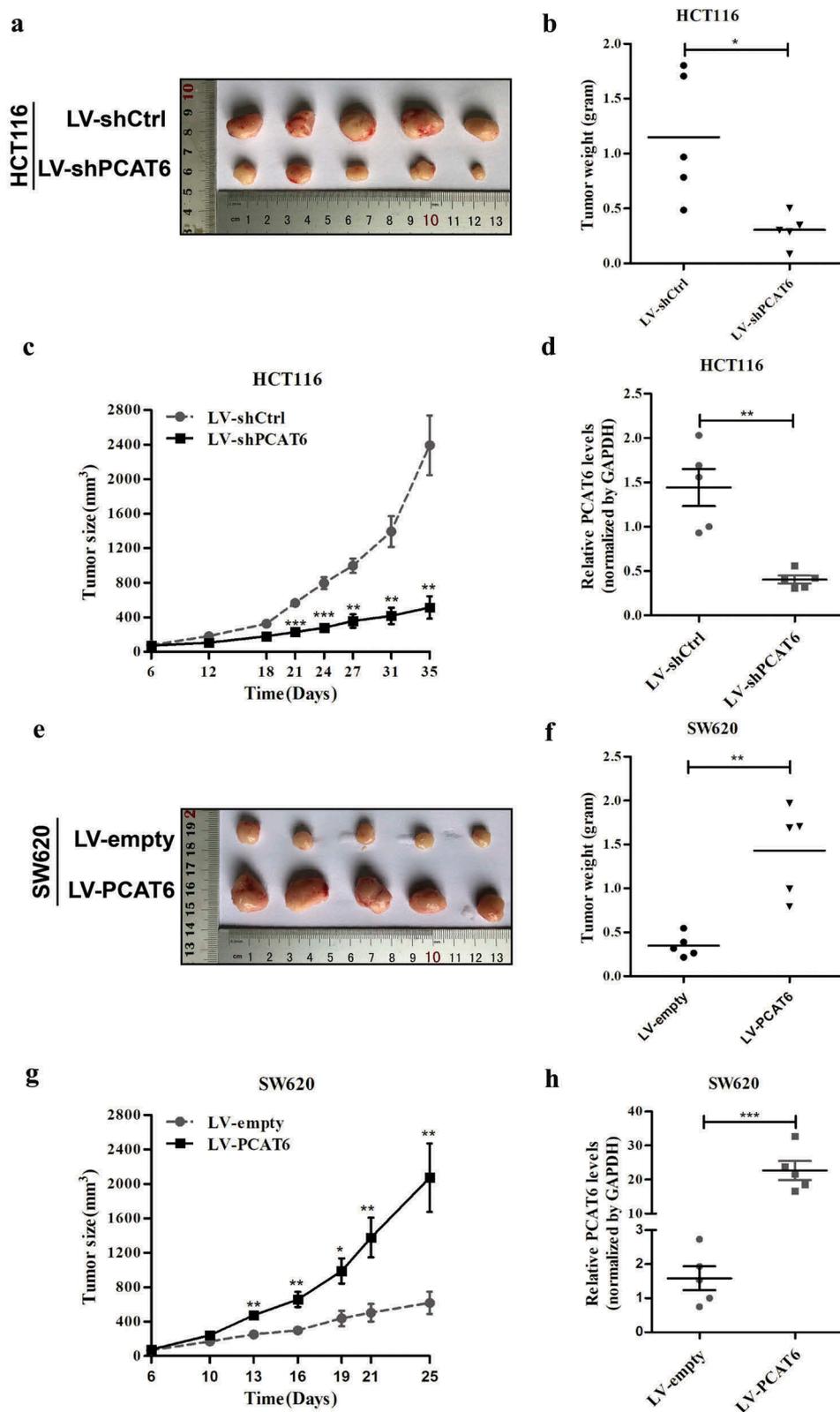
Figure 4. PCAT6 contributes to the tumorigenesis of colon cancer cells in vivo. (a-d) Effects of PCAT6 knockdown in HCT116 cells on subcutaneous tumor growth. (a) Representative images of xenograft tumors formed by HCT116 cells expressing control shRNA or shPCAT6 in nude mice. (b) The tumors were excised and weighed. (c) LV-shCtrl and LV-shPCAT6 cells were injected subcutaneously into nude mice and were assessed for tumor growth every 3 to 6 days. (d) qRT-PCR analysis of PCAT6 expression in xenograft tumors that were formed by HCT116-LV-shCtrl cells or HCT116-LV-shPCAT6 cells at day 35. (e-h) Effects of PCAT6 overexpression in SW620 cells on subcutaneous tumor growth. (e) Representative images of xenograft tumors formed by SW620 cells expressing exogenous PCAT6 or empty vector in nude mice, (e) and the tumor weights are shown. (g) The cells were injected subcutaneously into nude mice and assessed for tumor growth every 3 or 4 days. (h) qRT-PCR analysis of PCAT6 expression in xenograft tumors.

Together, these results indicated that PCAT6 plays a pivotal oncogenic role in colon cancer progression.

PCAT6 affects colon cancer cell apoptosis via regulating anti-apoptotic protein ARC expression

By determining PCAT6 expression via gene set enrichment analysis (GSEA) the Cancer Genome Atlas (TCGA) profiles, we found that PCAT6 levels were positively correlated with colon cancer growth by affecting genes in apoptosis regulation (Figure 5(a)). Our above study showed that PCAT6 had a profound influence on colon cancer cell apoptosis; Thus, we focused on the potential regulatory relationship between PCAT6 and apoptosis-related genes (HIP1, BBC3, IHPK2, NOL3, BCL2, APAF-1, FADD, PIM2, PIM1, and ZNF346) [18,19]. First, we conducted a correlation analysis to identify PCAT6's potential relevant apoptosis-related genes in the COAD-TCGA cohort. Interestingly, among those genes, we noticed that NOL3, which encodes an anti-apoptotic protein ARC, showed a significantly positive correlation with PCAT6 (Figure 5(b)). The previous study had demonstrated that ARC is present at high levels in almost all primary colon cancers compared with the corresponding controls [15]. Additionally, our study found that ARC had the same tendency in expression as PCAT6 in colon cancer cell lines (Supplementary Figure 1A-B). Furthermore, ARC was shown to decrease the enzyme activities of caspase 2 and caspase 8, triggering the inactivation of caspase 3 [14,20,21]. Based on these research foundations, we hypothesized that PCAT6 might regulate the expression of ARC. Thus, we first examined whether PCAT6 affects ARC expression in colon cancer cells. Our results showed that knocked down PCAT6 in colon cancer cells significantly decreased ARC expression at both the transcriptional and translation levels (Figure 5(c)), while upregulated PCAT6 increased ARC expression (Figure 5(d)). These results indicated that ARC might be a downstream target of PCAT6.

To test the functional roles of PCAT6 and ARC in colon cancer cells, we performed rescue assays. We first designed two shRNAs against ARC and confirmed the interference efficiency in colon cancer cells (Supplementary Figure 1C). Our data showed that the introduction of PCAT6 significantly promoted cell growth, while the knockdown of ARC by shRNAs rescued the PCAT6-induced increase in cell growth (Figure 5(e)). Additionally, the upregulation of PCAT6 inhibited cell apoptosis and decreased cleaved caspase 3, while knocked down ARC expression by shRNAs remarkably abolished the inhibition of exogenous PCAT6 on cell apoptosis and reversed the inactivation of PCAT6 on cleaved caspase 3 (Figure 5(f,g) and Supplementary Figure 1D).

Furthermore, in agreement with these observations, TCGA-COAD cohort transcriptome analysis revealed higher ARC mRNA in colon cancer tissues than non-cancerous tissues (Figure 5(h)). Kaplan–Meier analysis revealed high ARC mRNA was correlated with a worse overall survival (Figure 5(i)). Altogether, our results indicated that ARC is a functional downstream target of PCAT6 in colon cancer.

PCAT6 regulates ARC expression by interacting with EZH2 in colon cancer cells

We next explored the underlying molecular mechanisms by which PCAT6 mediates ARC expression in colon cancer cells. It has been proposed that the function of some lncRNAs is dependent on EZH2 [22]. By analyzing the data set of EZH2-interacting RNAs in HCT116 cells provided by Guil S [23], we identif-ied PCAT6 (RP11-480I12.3, ENST00000417262, Ensembl) as a potential EZH2- linked lncRNA. Indeed, our RNA immunoprecipitation assay using EZH2 antibody further verified the interaction of PCAT6 and EZH2 in HCT116 cells (Figure 6(a)). Therefore, we hypothesized that PCAT6 might regulate ARC expression via EZH2-mediated gene expression. To assess our hypothesis, we first designed two shRNAs against EZH2 to confirm whether EZH2 affects the expression of ARC. As expected, the results showed silenced EZH2 significantly reduced ARC expression at both the mRNA and protein levels in colon cancer cells, as well as increased the activation of cleaved caspase 3 (Figure 6(b)).

Previous analyzes performed by Rokavec M combining the chromatin immunoprecipitation (ChIP) assay with sequencing showed histone 3 lysine 4 trimethylation (H3K4me3) occupancy over the ARC genomic loci in colon cancer cells (Chr16:67207783–67208237) [24]. Moreover, the UCSC Genome database (http://genome.ucsc.edu/) indicated a potential binding site of EZH2 at the ARC genomic loci (chr16:67207781–67208904) that overlapped with H3K4me3 potential occupation site.
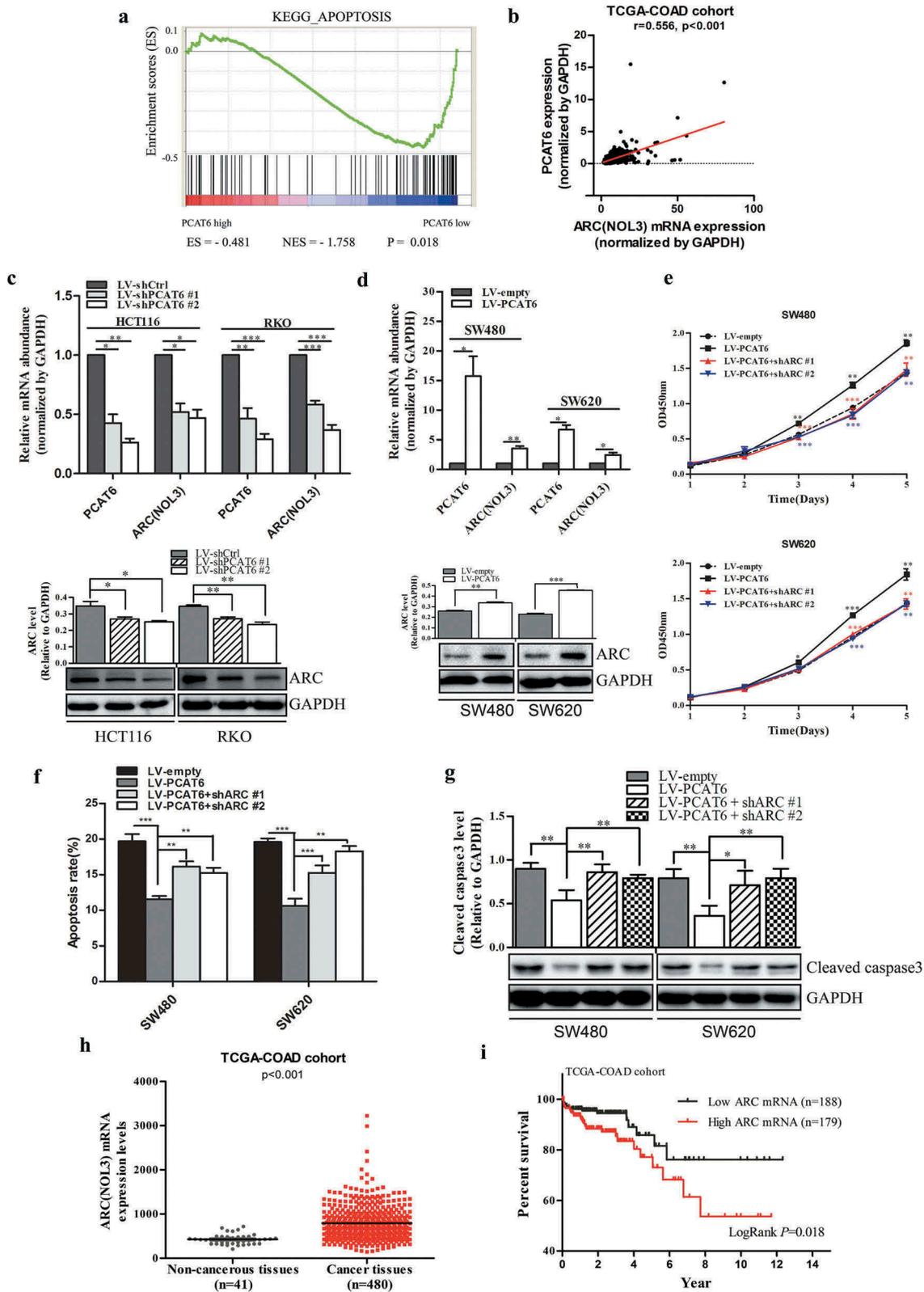
Figure 5. PCAT6 regulates anti-apoptotic protein ARC expression. (a) The GSEA plot showed that PCAT6 expression was correlated with the apoptosis gene signature. (b) Scatter plots of PCAT6 versus ARC mRNA expression in the TCGA-COAD data repository, and the Pearson correlation coefficient (r) and p value are shown. (c) Knockdown of PCAT6 expression decreased ARC expression in HCT116 and RKO cells (Up: ARC mRNA levels were measured by the qRT-PCR assay. Down: ARC protein levels were measured by the western blot assay). (d) Enforced PCAT6 expression increased ARC expression in SW480 and SW620 cells. (e) Enforced PCAT6 expression promoted cell growth, while knocked down ARC reversed the stimulation of PCAT6 on cell growth in SW480 and SW620 cells. (f) Upregulated PCAT6 expression decreased cancer cell apoptosis, and then knocked down ARC expression rescued the inhibition of PCAT6 on cell apoptosis in SW480 and SW620 cells. (g) Silencing ARC reversed PCAT6-induced inactivation of cleaved caspase 3. (h) ARC mRNA was highly expressed in colon cancer tissues. (i) Kaplan–Meier analysis of the correlation between ARC mRNA levels and overall survival in the TCGA-COAD cohort.
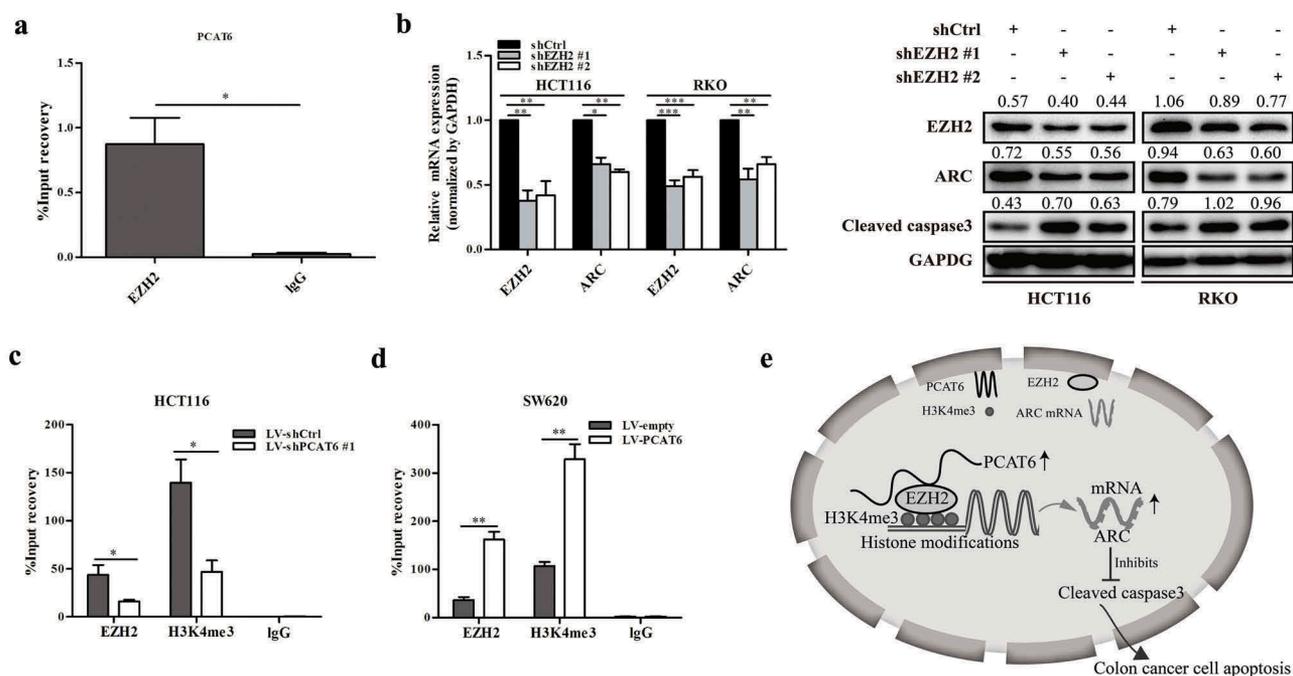
Figure 6. PCAT6 regulates ARC expression by interacting with EZH2. (a) RIP–qPCR assay was performed in HCT116 cells using the EZH2 antibody or normal rabbit IgG. (b) Silenced EZH2 reduced ARC expression and activated cleaved caspase 3. (c) Co-occupancy of EZH2 and H3K4me3 at the ARC genomic loci was decreased after knocking down PCAT6 in HCT116 cells; We used IgG as a negative control. (d) Co-occupancy of EZH2 and H3K4me3 at the ARC genomic loci was increased after elevating PCAT6 expression in SW620 cells. (e) Proposed model in which PCAT6 mediates the cell apoptosis of colon cancer.

Because H3K4me3 is critical for gene activation, while H3K9 and H3K27 methylation is associated with gene silencing [25], we speculated that EZH2 might act as an activator in ARC transcriptional activation but not as a suppressor. Next, we performed CHIP assays to verify our hypothesis. Consistent with our hypothesis, the results showed that the occupancy of EZH2 and H3K4me3 was significantly reduced across the ARC gene region in HCT116 cells upon the knockdown of PCAT6 (Figure 6(c)). By contrast, enforcing PCAT6 expression increased the binding of EZH2 and H3K4me3 across the ARC genomic region in SW620 cells (Figure 6(d)). Together, these data support a model whereby PCAT6 acts as a coactivator with EZH2 occupancy and activity to modulate ARC expression.

Discussion

The imbalance between cell growth and cell apoptosis represents one of the hallmarks of cancer treatment [13]. Therefore, investigating the molecular mechanisms that regulate cell death may provide helpful insights into the development of efficient diagnoses and therapeutic strategies of cancers. Recent studies have found that the dysregulated expression of lncRNAs in solid cancer was involved in regulating cancer cell apoptosis and may independently predict patient outcome [26]. To identify lncRNA signatures for colon cancer prognosis, we analyzed the expression pattern of lncRNAs using public databases. The expression pattern and a series of further experiments showed that PCAT6 is an oncogenic lncRNA in colon cancer. Moreover, we provide mechanistic insights into PCAT6 as an ARC expression activator via interacting with EZH2.

PCAT6 was reported to be aberrantly high expressed in prostate and lung cancer, promoting cancer cell proliferation and invasion [27]. In this study, we showed that PCAT6 is significantly upregulated in colon cancer tissues compared with that in noncancerous colon tissues. Importantly, high levels of PCAT6 in colon cancer tissues were correlated with poor clinical stage and worse survival status of colon cancer patients. Consistently, our further in vitro and in vivo studies showed that enforcing PCAT6 expression increased the cellular viability, while knockdown of PCAT6 repressed cellular viability. Our results

illustrated that PCAT6 had no significant effect on cell cycle arrest but remarkably inhibited cell apoptosis and cleaved caspase 3 activation in colon cancer cells. Mechanistically, PCAT6 and anti-apoptotic protein ARC showed a positive correlation in the gene expression cohorts of colon cancer. It was confirmed that ARC acted as an endogenous inhibitor of apoptosis, directly interacted with Fas, FADD, and procaspase-8, which interfered with DISC assembly, as well as suppressed Bax and P53-mediated cell apoptosis [14,21,28]. Our further experiments showed that silencing PCAT6 decreased the expression of ARC in colon cancer, while upregulating PCAT6 promoted ARC expression. Furthermore, we demonstrated that knockdown ARC efficiently promoted colon cancer cell apoptosis and increased cleaved caspase3. Because the expression of ARC was controlled by PCAT6 in colon cancer cells, one critical question was then raised: What was the mechanism by which PCAT6 regulated ARC expression?

It is well known that EZH2 functions as a histone H3 Lys 27 (H3K27) methyltransferase when part of the Polycomb-repressive complex 2 (PRC2), acting as a gene expression repressor by catalyzing the addition of methyl groups onto lysine 27 of histone H3 (H3K27me2/3) [22]. However, several studies also identified EZH2 activated gene transcription by associating with the active histone mark H3K4me3 [29–31]. Sufficient evidence has shown that lncRNAs are detected to interact with EZH2 and regulate gene transcription [23]. In this study, using bioinformatics analyzes and published articles, PCAT6 was found to be a potential EZH2-linked RNA in HCT116 cells [23]. Moreover, EZH2 and H3K4me3 have an overlapped binding site at ARC genomic loci [24]. Therefore, we speculated the modulation of ARC expression by PCAT6 probably via EZH2-mediated gene activation. Next, we performed a series of experiments to verify our speculation. First, we confirmed the interaction between PCAT6 and EZH2 using the RIP-qPCR assay. The results showed that PCAT6 was enriched in the EZH2 antibody pull-down products compared with the IgG control in HCT116 cells. Second, we used two EZH2 shRNAs to confirm the role of EZH2 in ARC expression. Our experiments showed that silencing EZH2 inhibited ARC expression at both the transcription and translation levels in colon cancer cells, indicating that EZH2 is

a functional upstream molecule of ARC. Third, the CHIP-qPCR assay showed that silencing PCAT6 decreased the binding of EZH2 and H3K4me3 levels across the ARC genomic region, while the overexpression of PCAT6 increased the binding of EZH2 and H3K4me3 levels across the ARC genomic region in colon cancer cells. Taken together, we showed a model whereby PCAT6 acts as a coactivator with EZH2 and H3K4me3 occupancy and modulates ARC expression.

In conclusion, our results provide a new perspective for PCAT6 acting as a noncoding oncogene in colon cancer tumorigenesis and might be a novel predictor for colon cancer patients. Furthermore, PCAT6 acts as a key regulator of the anti-apoptotic protein ARC expression. Additionally, PCAT6 can be associated with EZH2 and regulates EZH2 mediated gene expression. These findings suggest that PCAT6 plays a role in the development and progression of colon cancer, rendering PCAT6 a potential prognostic marker that may serve as a novel therapeutic target of patients with colon cancer.

Disclosure statement

No potential conflict of interest was reported by the authors.

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