Article

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FABP4 Regulates Cell Proliferation, Stemness, Apoptosis, and Glycolysis in Colorectal Cancer via Modulating ROS/ERK/mTOR Pathway

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Background: Colorectal cancer is a common digestive tract malignancy. This study aimed to expound the functional role of fatty-acid-binding protein 4 (FABP4) and the potential underlying mechanisms in the development of colorectal cancer.

Methods: Several techniques were utilized to investigate the role of FABP4 in colorectal cancer. FABP4 mRNA expression was quantified using Real time-quantitative PCR (RT-qPCR). Cell counting kit-8 (CCK-8), 5-ethynyl-2'-deoxyuridine (EdU), sphere formation assays and flow cytometry evaluated cell growth, stemness, and apoptosis in SW480 and HT29 cells. Glycolysis was assessed via extracellular acidification rate (ECAR) , lactate production, glucose uptake, adenosine triphosphate (ATP)/adenosine 5'-diphosphate (ADP) ratio, and Glut1 and Elevated lactate dehydrogenase A (LDHA) protein expression. Reactive oxygen species (ROS) levels were analyzed by flow cytometry. Western blot measured the protein expression of FABP4, Proliferating cell nuclear antigen (PCNA), Bax, Bcl-2, Glut1, LDHA, stemness makers (Sox2, Oct4, and ALDHA1), and extracellular regulated protein kinase (ERK)/mammalian target of rapamycin (mTOR) pathway proteins. *In vivo* experiments, BALB/c nude mice (n = 12) were inoculated with 200 μL HT29 cells (5×10^6 cells) transfected with sh-FABP4 or short hairpin (sh)-negative control (NC), forming two groups with 6 mice each. The *in vivo* mice tumor model allowed for evaluating FABP4's impact on tumor growth.

Results: FABP4 was significantly upregulated in colorectal cancer tissues and cells ($p < 0.05$). FABP4 knockdown markedly inhibited cell proliferation, stemness, and glycolysis, while promoting apoptosis in these cells ($p < 0.05$). Additionally, FABP4 depletion led to a significant increase in ROS level ($p < 0.05$). However, N-acetyl-L-cysteine (NAC) ($p < 0.05$), a ROS scavenger, mitigates these effects. Furthermore, the effects of FABP4 depletion on cell growth, stemness, glycolysis, and apoptosis in colorectal cancer cells were also retarded by NAC ($p < 0.05$). Notably, FABP4 knockdown also suppressed the ERK/mTOR pathway, suggesting its regulation via ROS ($p < 0.05$). *In vivo* study results showed, FABP4 depletion significantly curbed tumor growth in colorectal cancer ($p < 0.05$).

Conclusions: These results suggest that FABP4 depletion inhibits colorectal cancer progression by modulating cell growth, stemness, glycolysis and apoptosis. This regulation occurs through the ROS/ERK/mTOR pathway.

Keywords: colorectal cancer; FABP4; NAC; ERK/mTOR pathway

Introduction

Colorectal cancer is a common digestive department tumor and its mortality and morbidity rank third among all global tumors [1–3]. Recent reports indicate a steady increase in its incidence [4,5], with associated death reaching 900,000 globally in 2020 [6]. Despite the widespread use of systemic chemotherapy, radiotherapy, and surgical resection in clinical practice to enhance survival rates [7], the prognosis remains poor. Even with significant advancements in comprehensive therapy and clinical diagnosis, the five-year survival rate is less than 50% due to factors like distant metastasis and chemoresistance [1,8,9]. Previous studies have indicated the role of both environmental and genetic factors in colorectal cancer's development and progression [10,11]. Cancer stem cells (CSCs), characterized by resistance to apoptosis, asymmetric cell division, self-renewal, high metastatic potential, and tumorigenicity, are key players in tumor growth, chemoresistance, and metastasis [12–15]. Thus, exploring molecules that regulate the

stemness of colorectal cancer may help identify promising therapeutic targets for this disease.

Fatty acid binding proteins (FABPs) are known for binding and transporting hydrophobic fatty acids. FABP4, a member of the FABPs family, is predominantly found in dendritic cells, macrophages, adipocytes, and microvascular endothelial cells [16,17]. It plays vital roles in intracellular signal transduction, metabolism, and lipid transport, influencing diseases like atherosclerosis, inflammation, type 2 diabetes, and metabolic syndrome [18–21]. While one study found FABP4 protein level significantly downregulated in liver cancer, correlating negatively with tumor stage [22], others reported increased FABP4 levels in certain tumors [23–25]. Recently, the expression of FABP4 in colorectal cancer was also excavated, and the data showed that FABP4 was overexpressed in colorectal cancer [17,26]. However, the precise mechanisms by which FABP4 contributes to colorectal cancer development remain unclear.

Reactive oxygen species (ROS) are oxygen-containing molecules which naturally produced by cell metabolism [27]. Researches have disclosed that the modulation of ROS production participated in multiple aspects of cancer development, such as drug resistance development, aggressive phenotypes, metabolic reprogramming, and carcinogenesis [28]. Elevated ROS levels in tumor cells can be toxic, potentially triggering cell death pathways, apoptosis, and cell cycle arrest [29,30]. High ROS levels can promote tumor cell growth [31]. ROS notably influences the MAPK/ERK pathway, associated with cell differentiation, metastasis, stress responses, and growth [32]. Abnormal ERK activation can cause DNA damage and apoptosis [33]. Furthermore, mTOR, modulated by the MAPK/ERK pathway, is crucial for cancer survival and autophagy [34–36].

This study investigated whether FABP4 affects cell growth, stemness, apoptosis, and glycolysis in colorectal cancer through the ROS/ERK/mTOR pathway. We knocked down FABP4 expression to determine its function in colorectal cancer progression. Our findings indicate that FABP4 can influence cell growth, stemness, apoptosis, and glycolysis through the ROS-mediated ERK/mTOR pathway in colorectal cancer.

Material and Methods

Tissue Specimens

Sixty-six patients with colorectal cancer undergoing surgery at the First Hospital of Hebei Medical University graciously provided both tumors and matched adjacent normal specimens (n = 66). Immediately after collection, these samples were promptly stored at –80 °C to preserve their integrity for future analyses. Crucially, prior to this study, none of the participants had received any form of preoperative anti-cancer therapies. For reference, the clinicopathological parameters of colorectal cancer are listed in Table 1.

Table 1. The clinicopathological parameters of the patients with colorectal cancer.

Variable	Patients, n
Age, years	66
<65	37
≥65	29
Gender	
Male	34
Female	32
TNM stage	
I+II stage	32
III+IV stage	34
History of colon polyps	
Yes	35
No	31

Cell Culture and Transfection

SW480, HT29, and NCM460 cells were cultured in DMEM medium (Gibco, Carlsbad, CA, USA) containing 100 U/mL penicillin (Invitrogen, Carlsbad, CA, USA), 100 U/mL streptomycin (Invitrogen), and 10% FBS (Invitrogen). These cells were cultured under specific conditions in a humidified incubator with a controlled atmosphere of 5% CO_2-95% O_2, maintained at a temperature of 37 °C. All cell lines underwent stringent mycoplasma testing and STR validation procedures. As a reactive oxygen species (ROS) scavenger, N-acetyl-L-cysteine (NAC; 2 mM, MedChemExpress, Shanghai, China) was employed to treat the HT29 and SW480 cells [37].

In order to suppress FABP4 expression, siRNA targeting FABP4 (si-FABP4: sense, 5′-GACUUCCACAAGAGUUUAUTT-3′, antisense, 5′-AUAAACUCUUGUGGAAGUCTT-3′) sequence was designed by Genepharma (Shanghai, China). This was subsequently introduced into the SW480 and HT29 cells, utilizing si-NC (sense, 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense, 5′-ACGUGACACGUUCGGAGAATT-3′) as a negative control for comparison. The transfection process was facilitated using Lipofectamine 3000 Reagent (Invitrogen), ensuing transient gene delivery within the cells.

RT-qPCR

The Trizol kit (Invitrogen) was employed to isolate the total RNA, which was subsequently converted into complementary DNA (cDNA) using the RT-PCR kit (Promega, Madison, WI, USA) in accordance with the protocol provided by the manufacturer. Afterwards, a real-time quantitative PCR system (Bio-Rad, Hercules, CA, USA) was applied for qPCR. To evaluate the relative expression levels of FABP4, we used the $2^{-\Delta\Delta Ct}$ method, with GAPDH serving as an internal control for normalization. The primer used in this study are as follows: for GAPDH, 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward) and

5′-TGGTGAAGACGCCAGTGGA-3′ (reverse); and for FABP4, 5′-TGGGCCAGGAATTTGACGA-3′ (forward) and 5′-CATTTCTGCACATGTACCAGGACAC-3′ (reverse).

Cell Proliferation

Cell viability and proliferation were assessed through CCK-8 and Edu assays. Using a CCK-8 kit (Dojindo, Tokyo, Japan), a cell suspension of 2×10^3 cells was incubated with CCK-8 solution for 2 hours, after which absorbance at 450 nm was measured to evaluate cell viability. Concurrently, an EdU assay was conducted using an EdU kit (Beyotime, Shanghai, China). A separate batch of cells (5×10^3 cells) was stained with EdU reagent for 2 hours, followed by a 15-minute DAPI treatment to re-stain the nuclei. Following staining, the cells were examined under a fluorescence microscope (Olympus, Tokyo, Japan) to detect the presence of Edu-positive cells, which serve as cell proliferation markers. Together, these two assays comprehensively assessed the cell proliferation capacity.

Sphere Formation Assay

A total of 1000 SW480 or HT29 cells were seeded into an ultralow attachment plate. These cells were nourished with serum-free RPMI-1640 medium, which was enhanced with EGF (20 ng/mL; Gibco), 1% B27 (Gibco), and FGF (20 ng/mL; Gibco). Following a 2-week incubation period, spheroid clones formed. The size of these spheroids was then estimated using Image J software (version 1.8.0, LOCI, University of WisconsinUniversity of Wisconsin, Madison, WI, USA), and a microscope was used to count the total number of spheres.

Flow Cytometry

Upon collection, treated SW480 and HT29 cells (5×10^4) were stained in darkness with 10 μL of reagent mixture containing propidium iodide (PI; 5 μL; Beyotime) and Annexin V-fluorescein isothiocyanate (FITC; 5 μL; Beyotime). The level of apoptosis was then evaluated using flow cytometry.

Western Blot

Total proteins were isolated using RIPA buffer and separated via 10% SDS-PAGE. These proteins were then transferred to PVDF membranes, which were blocked with 5% skimmed milk to prevent non-specific binding. Then, the membranes were incubated with the primary antibodies at 4 °C overnight and the secondary antibody for 1 h at room temperature. The antibodies were PCNA (ab18197; 1:1000; Abcam, Cambridge, UK), Bax (ab32503; 1:1000; Abcam), FABP4 (ab66682; 1:1000; Abcam), Bcl-2 (ab32124; 1:1000; Abcam), Sox2 (ab97959; 1:1000; Abcam), Oct4 (ab19857; 1:1000; Abcam), ALDHA1 (15910-1-AP; 1:1000; Proteintech, Wuhan, China), Glut1 (ab15309; 1:1000; Abcam), LDHA (ab52488; 1:1000; Abcam), p-ERK (4370; 1:1000; Cell Signaling Technology, Boston, MA, USA), ERK (4695; 1:1000; Cell Signaling Technology), p-mTOR (5536; 1:1000; Cell Signaling Technology), mTOR (2983; 1:1000; Cell Signaling Technology), and GAPDH (ab181602; 1:1000; Abcam). Finally, the enhanced chemiluminescence kit (Millipore, Billerica, MA, USA) was conducted to obtain the combined protein signals.

Extracellular Acidification (ECAR)

After treatment, SW480 and HT29 cells (2×10^4 per well) were seeded into the XF96-well plate. Firstly, glucose was added to these cells, and then oxidative phosphorylation inhibitor (oligomycin) and glycolytic inhibitor (2-deoxyglucose; 2-DG) were sequentially plus into the culture medium containing the treated cells. ECAR was calculated using Seahorse XF-96 Wave software (version 1.4.2.3, Seahorse Bioscience; Agilent Technologies, Inc., Santa Clara, CA, USA).

Measurement of Lactate Production, Glucose Uptake, and ATP/ADP Ratio

The levels of lactate production, ATP/ADP ratio, and glucose uptake were assessed using respective kits- a Lactate Assay Kit, an ATP/ADP Ratio Assay kit, and a Glucose Assay Kit, all from BioVision (Milpitas, CA, USA). Each of these assessments was conducted following the respective manufacturer's protocol.

Detection of Reactive Oxygen Species (ROS) Level

After removing the medium and washing the cells, DCFH-DA (10 μM) was introduced into each well. The cells were then incubated for 30 minutes at 37 °C in the dark. Post-incubation, the treated SW480 and HT29 cells were trypsinized, collected, and immediately subjected to analysis using FACSCalibur flow cytometry (BD FACSCalibur flow cytometer, FACS101; BIO-RAD Corporation, Hercules, CA, USA).

Tumor Model

5-week-old BALB/c nude mice (n = 12) were sourced from Beijing Vital River Laboratory Animal Technology (Beijing, China) to create the murine xenograft model. These mice were subcutaneously injected with 200 μL HT29 cells (5×10^6 cells) stably transfected with either sh-FABP4 or sh-NC. The mice were evenly divided into the sh-FABP4 group (n = 6) and the sh-NC group (n = 6). The progression of tumor growth was regularly tracked by taking tumor size measurements every seven days. All mice were executed using the euthanasia method. The Animal Ethics Committee at the First Hospital of Hebei Medical University authorized the research protocol, with the approval number MDL 2022-05-15-03.

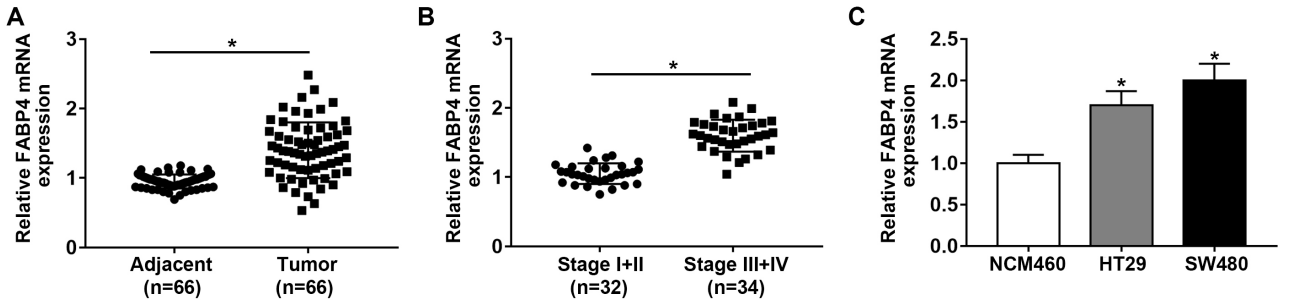

Fig. 1. Significant upregulation of FABP4 in colorectal cancer tissues and cells. (A) Detection of FABP4 mRNA expression in colorectal cancer specimens (n = 66) and the adjacent normal specimens (n = 66) using RT-qPCR. (B) Measurement of FABP4 mRNA expression in various TNM stage (I+II (n = 32), III+IV (n = 34) colorectal cancer patients via RT-qPCR. (C) Assessment of FABP4 mRNA expression in NCM460, HT29, and SW480 cells through RT-qPCR (n = 3). *$p < 0.05$.

Statistical Analysis

Each experiment was independently repeated three times. Data were shown as means ± standard deviation. Differences were analyzed via Student's *t*-test and ANOVA, with $p < 0.05$ deemed statistically significant.

Results

Significant Upregulation of FABP4 in Colorectal Cancer Tissues and Cells

We began by assessing the expression level of FABP4 in colorectal cancer tissues. Notably, we found a higher level of FABP4 expression in colorectal cancer tissues (n = 66) compared to adjacent normal tissues (n = 66) ($p < 0.05$) (Fig. 1A). Furthermore, FABP4 expression was significantly higher in the advanced stages (stages III and IV, also referred to as advanced tumor node metastasis stage) relative to the earlier stages (stages I and II) ($p < 0.05$) (Fig. 1B). Mirroring this trend, we observed an increased mRNA level of FABP4 in colorectal cancer cell lines (SW480 and HT29 cells) in contrast to NCM460 cells ($p < 0.05$) (Fig. 1C).

FABP4 Depletion Restrained Cell Proliferation, Stemness, and Triggered Apoptosis in Colorectal Cancer Cells

As demonstrated in Fig. 2A, si-FABP4 effectively knocked down FABP4 expression in both SW480 and HT29 cells ($p < 0.05$), evidencing its successful inhibition efficiency. Subsequently, we observed a significant reduction in cell viability when FABP4 was downregulated in these colorectal cancer cells ($p < 0.05$) (Fig. 2B). This downregulation also led to a drastic decrease in the number of EdU-positive cells in SW480 and HT29 cells transfected with si-FABP4 ($p < 0.05$) (Fig. 2C). Moreover, the sphere formation assay disclosed a notable reduction in the number of spheres formed following FABP4 knockdown in SW480 and HT29 cells ($p < 0.05$) (Fig. 2D). It was proved that the apoptosis of SW480 and HT29 cells was dramatically boosted after FABP4 depletion ($p < 0.05$) (Fig. 2E). In the subsequent examination of protein expression by western blot, we observed the influence of FABP4 depletion on proliferation-associated protein (PCNA), stemness markers (Sox2, Oct4, and ALDHA1), and apoptosis-related proteins (Bax and Bcl-2). Our results showed that FABP4 knockdown significantly decreased PCNA and Bcl-2 expression ($p < 0.05$) while increasing Bax expression ($p < 0.05$) (Fig. 2F). Moreover, we found that FABP4 knockdown led to a reduction in the levels of stemness markers, including Sox2, Oct4, and ALDHA1, in SW480 and HT29 cells ($p < 0.05$) (Fig. 2G).

FABP4 Depletion Inhibited Glycolysis in Colorectal Cancer Cells

Given the crucial role that glycolysis plays in cancer development, we investigated the potential influence of FABP4 on glycolysis in colorectal cancer. Extracellular Acidification Rate (ECAR) was utilized to indicate glycolysis, reflecting the overall glycolytic flux. Our findings suggest that the downregulation of FABP4 could significantly inhibit ECAR in both HT29 (Fig. 3A,B) and SW480 (Fig. 3C,D) cells ($p < 0.05$). Additionally, the downregulation of FABP4 considerably reduced lactate production, glucose uptake, and ATP/ADP ratio in colorectal cancer cells ($p < 0.05$) (Fig. 3E–G). To further understand the impact on glycolysis, we examined the levels of glycolysis-related proteins (LDHA and Glut1). The results confirmed that FABP4 knockdown dramatically lowered the protein levels of Glut1 and LDHA in colorectal cancer cells ($p < 0.05$) (Fig. 3H).

FABP4 Depletion Enhanced ROS Level in Colorectal Cancer Cells

Our observations revealed a significant increase in the levels of ROS when FABP4 was downregulated in both HT29 and SW480 cells ($p < 0.05$). However, this increase in ROS was restored by the application of NAC ($p < 0.05$), a known ROS scavenger (Fig. 4). These results indicated

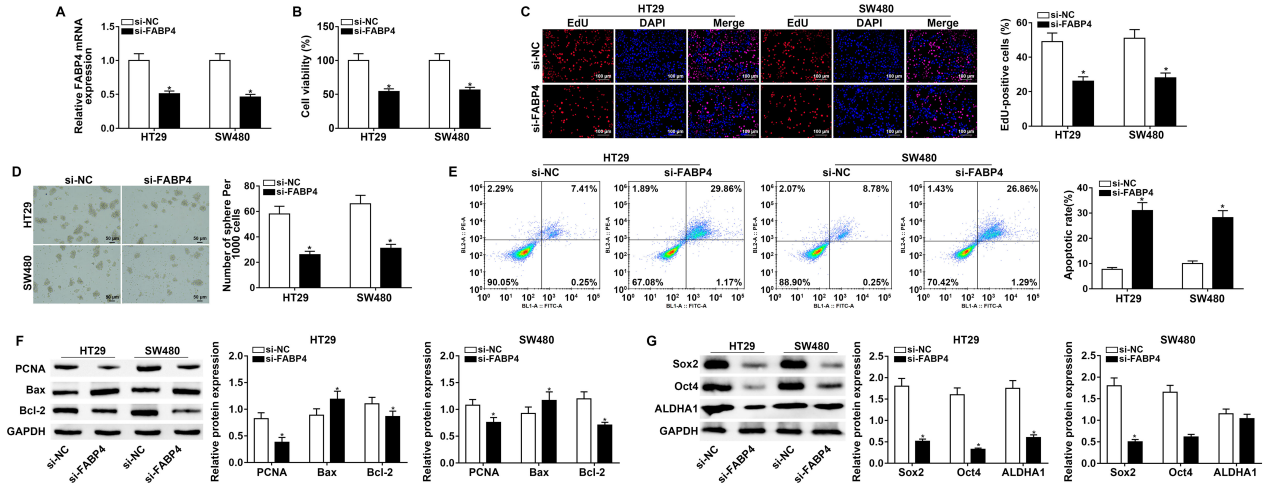

Fig. 2. FABP4 depletion restrained cell proliferation, stemness, and triggered apoptosis in colorectal cancer cells. (A–G) SW480 and HT29 cells were transfected with si-NC or si-FABP4. (A) The Knockdown efficiency of si-FABP4 was evaluated using RT-qPCR (n = 3). (B,C) CCK-8 and EdU assays were used for detecting cell proliferation (n = 3). (D) Sphere formation assay for stemness evaluation (n = 3). (E) Flow cytometry for apoptosis detection (n = 3). (F) Western blot for PCNA, Bax, and Bcl-2 protein expression measurement (n = 3). (G) Western blot was conducted to assess the expression of Sox2, Oct4, and ALDHA1 (n = 3). *$p < 0.05$.

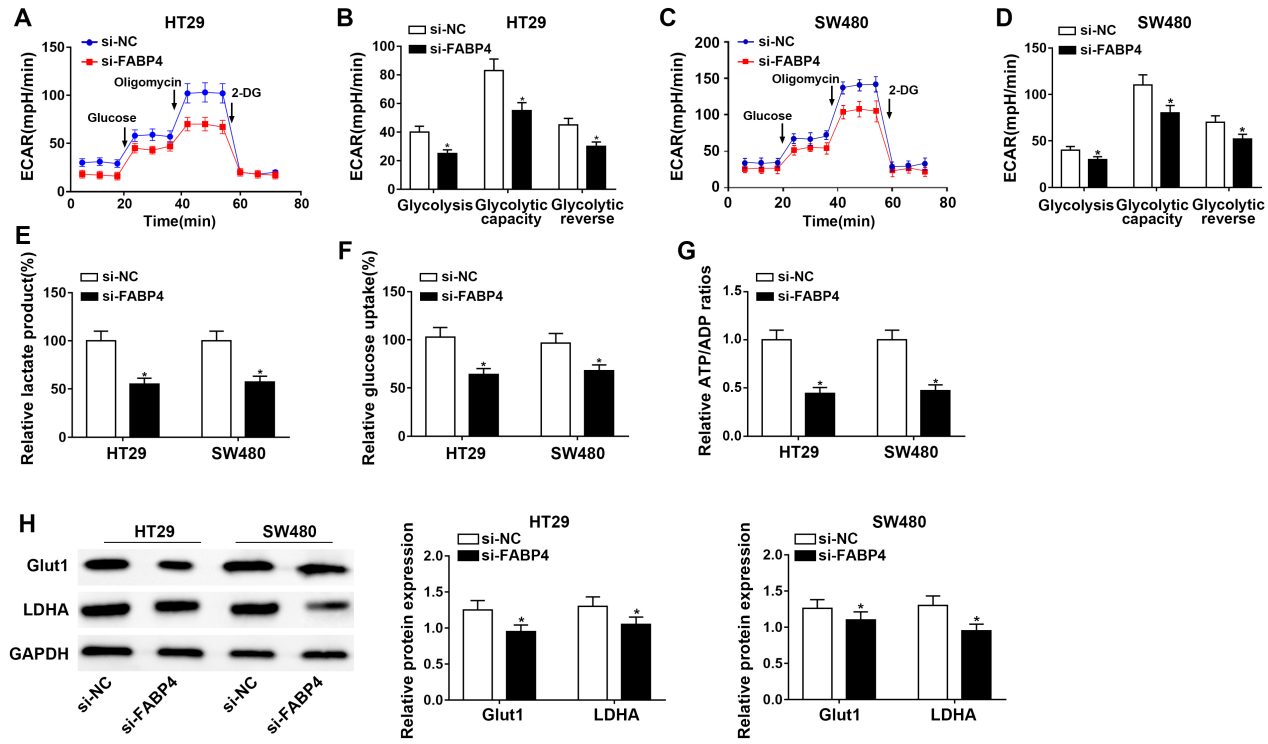

Fig. 3. FABP4 depletion inhibited glycolysis in colorectal cancer cells. (A–H) SW480 and HT29 cells were transfected with si-NC or si-FABP4. (A–D) ECAR was quantified in SW480 and HT29 cells using Seahorse Extracellular Flux Analyzer XF96 assays (n = 3). (E–G) Determination of lactate release (E), glucose uptake (F), and ATP/ADP ratio (G) using the corresponding kits (n = 3). (H) Glut1 and LDHA protein expression were detected using western blot (n = 3). *$p < 0.05$.

that NAC could reverse the effect of FABP4 depletion on ROS level in colorectal cancer cells.

NAC Mitigates the Effect of FABP4 Depletion on Colorectal Cancer Progression

Subsequently, we delved into whether the depletion of FABP4 influenced cellular processes such as proliferation,

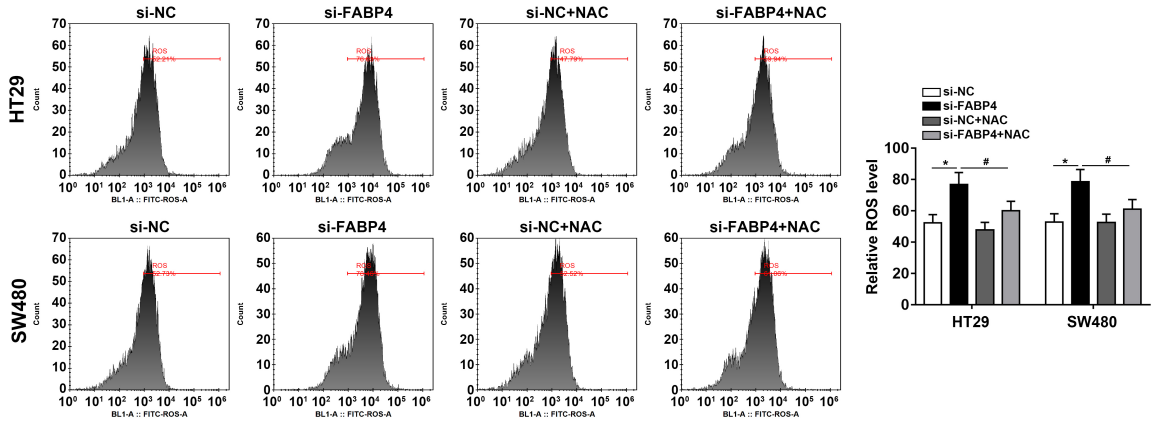

Fig. 4. FABP4 depletion enhanced ROS level in colorectal cancer cells. Measurement of ROS level in SW480 and HT29 cells treated with si-NC, si-FABP4, si-NC+NAC, or si-FABP4+NAC; NAC was a ROS scavenger (n = 3). *$p < 0.05$ vs si-NC; #$p < 0.05$ vs si-FABP4.

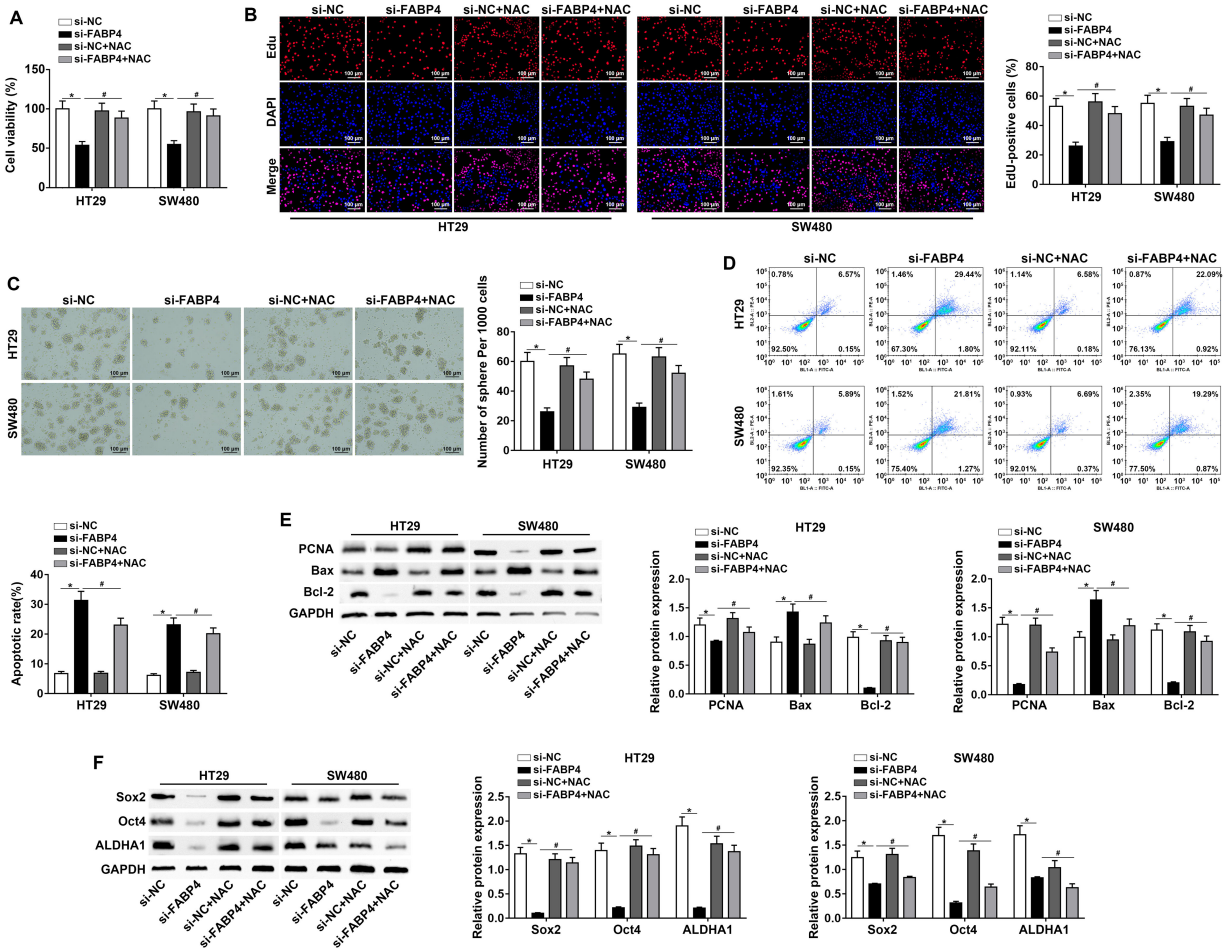

Fig. 5. NAC mitigates the effect of FABP4 depletion on colorectal cancer progression. (A–F) SW480 and HT29 cells were treated with si-NC, si-FABP4, si-NC+NAC, or si-FABP4+NAC. (A–D) Determination of cell proliferation, stemness, and apoptosis using CCK-8, EdU, sphere formation assays, and flow cytometry, respectively (n = 3). (E) Measurement of PCNA, Bax, and Bcl-2 protein expression via western blot (n = 3). (F) Sox2, Oct4, and ALDHA1 protein expression were detected via western blot (n = 3). *$p < 0.05$ vs si-NC; #$p < 0.05$ vs si-FABP4.

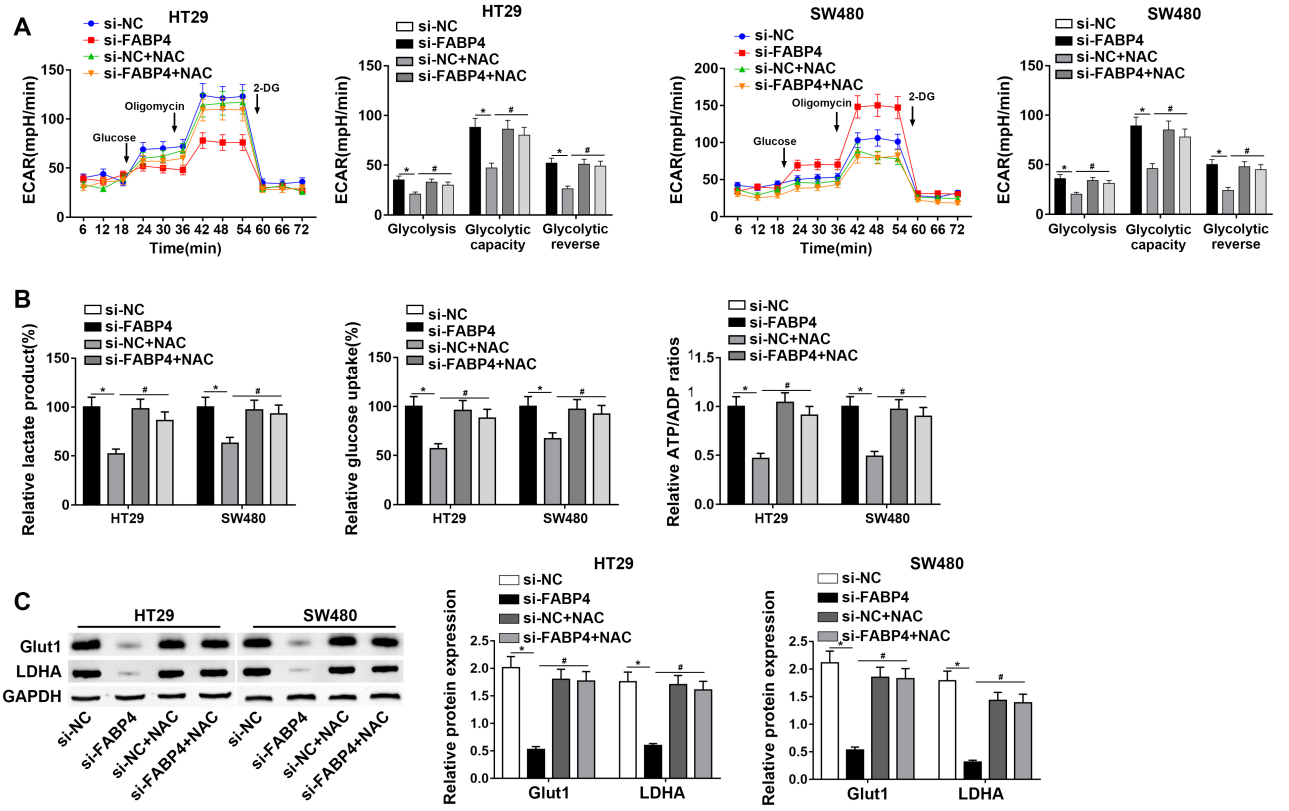

Fig. 6. NAC potentially mitigates the inhibition of glycolysis triggered by FABP4 depletion. (A–C) SW480 and HT29 cells were treated with si-NC, si-FABP4, si-NC+NAC, or si-FABP4+NAC. (A) Analysis of ECAR in SW480 and HT29 cells (n = 3). (B) Assessment of lactate release, glucose uptake, and ATP/ADP ratio using the corresponding kits (n = 3). (C) Western blot detected Glut1 and LDHA protein levels (n = 3). *$p < 0.05$ vs si-NC; #$p < 0.05$ vs si-FABP4.

stemness, apoptosis, and glycolysis by regulating the ROS level. The results disclosed that the repressive impacts of FABP4 knockdown on cell proliferation (Fig. 5A,B), stemness (Fig. 5C), and the promotion impact on cell apoptosis (Fig. 5D) were reversed by NAC treatment in SW480 and HT29 cells ($p < 0.05$). This was further substantiated by western blot analysis, which showed increased expression of PCNA and Bcl-2, decreased Bax expression, and elevated Sox2, Oct4, and ALDHA1 in SW480 and HT29 cells ($p < 0.05$) (Fig. 5E,F). Furthermore, the inhibitory impact of FABP4 knockdown on glycolysis was mitigated by NAC treatment, as evidenced by increased ECAR (Fig. 6A), elevated lactate production, glucose uptake, and ATP/ADP ratio (Fig. 6B), and upregulated expression of Glut1 and LDHA (Fig. 6C) in SW480 and HT29 cells ($p < 0.05$).

FABP4 Depletion Activated the ERK/mTOR Signaling Pathway via Modulating the ROS

Previous studies reported that ERK/mTOR pathway was involved in the progression of types of cancers [34,38]. As Fig. 7A demonstrates, FABP4 depletion notably heightened the expression of p-ERK in SW480 and HT29 cells ($p < 0.05$), whereas FABP4 knockdown led to a reduction in p-mTOR expression ($p < 0.05$). This suggests that FABP4 depletion could activate the ERK/mTOR signaling pathway. Furthermore, the ERK/mTOR pathway activation induced by FABP4 knockdown was inhibited upon treatment with NAC ($p < 0.05$) (Fig. 7B). This suggests that FABP4 depletion can potentially activate the ROS/ERK/mTOR pathway in colorectal cancer cells.

FABP4 Depletion Repressed Tumor Growth through the ERK/mTOR Pathway

Our data indicate that the volume of the tumor was significantly reduced following FABP4 depletion ($p < 0.05$) (Fig. 8A). Furthermore, protein expression of FABP4 was notably decreased upon FABP4 knockdown in nude mice ($p < 0.05$) (Fig. 8B). The ERK/mTOR pathway was also inactivated by FABP4 depletion, as identified by reducing p-ERK/ERK ratio and increasing p-mTOR/mTOR ratio ($p < 0.05$) (Fig. 8C).

Discussion

We elucidated the role of the FABP4/ROS/ERK/mTOR axis in regulating cell growth, stemness, glycolysis, and apoptosis in colorectal cancer. Our results highlighted an elevated presence of FABP4 in

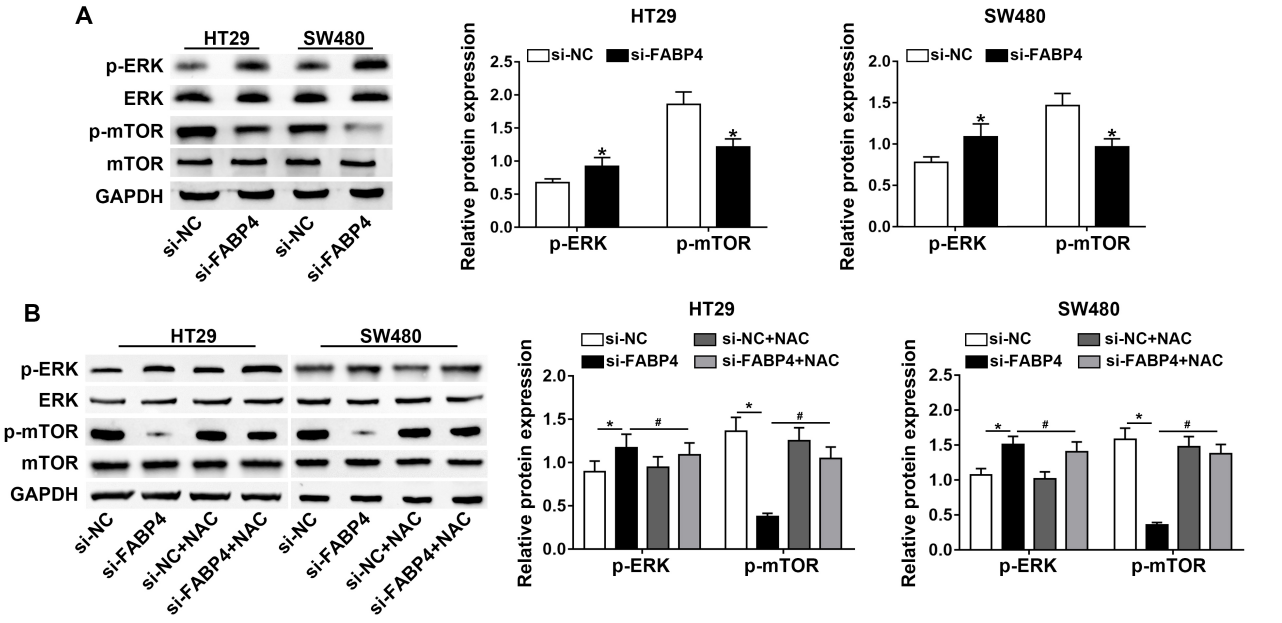

Fig. 7. FABP4 depletion activated the ERK/mTOR signaling pathway via modulating the ROS. (A) The protein expression of ERK, p-ERK, mTOR, and p-mTOR in SW480 and HT29 cells transfected si-NC or si-FABP4 was detected using western blot (n = 3). (B) Measurement of ERK, p-ERK, mTOR, and p-mTOR protein expression in SW480 and HT29 cells treated with si-NC, si-FABP4, si-NC+NAC, or si-FABP4+NAC using western blot (n = 3). *$p < 0.05$ vs si-NC; #$p < 0.05$ vs si-FABP4.

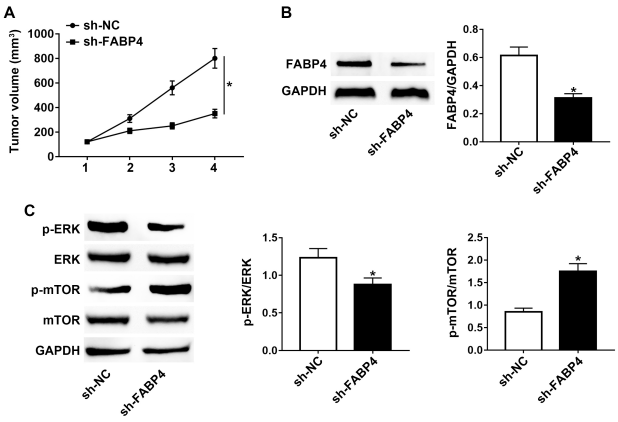

Fig. 8. FABP4 depletion repressed tumor growth through the ERK/mTOR pathway. (A) Determination of tumor volume to monitor tumor growth (n = 6). (B) The protein expression of FABP4 was measured by western blot (n = 3). (C) The protein expressions of ERK/mTOR pathway-related proteins were determined by western blot (n = 3). *$p < 0.05$.

colorectal cancer. Notably, FABP4 depletion significantly curtailed cell proliferation, stemness, and glycolysis while simultaneously enhancing cell apoptosis and ROS levels. Moreover, the inhibitory effects of FABP4 depletion on cell growth, stemness, glycolysis, and apoptosis, alongside the elevation of ROS levels, were mitigated by NAC, a ROS scavenger. Importantly, FABP4 depletion was found to disrupt the activation of the ERK/mTOR pathway through ROS modulation. *In vivo* experiment results further corroborated the repressive role of FABP4 depletion in the progression of colorectal cancer by modulating the ERK/mTOR pathway. These findings suggest that FABP4 depletion-induced ROS impacts cell glycolysis, proliferation, stemness, and apoptosis in colorectal cancer by inactivating the ERK/mTOR pathway.

The FABP family encompasses at least 12 family members, and all are acknowledged as intracellular lipid chaperones [39]. Despite the tissue-specific diversity of FABP isoforms, each demonstrates significant biological functions [40,41]. The impact of FABP4 varies considerably, sometimes paradoxically, among different tumors. One study reported overexpression of FABP4 promoting cholangiocarcinoma cell metastasis and facilitating intracellular lipid accumulation [42]. Conversely, in omental ovarian cancer metastases, FABP4 was substantially increased yet remained unchanged in primary tumors [18]. In prostate cancer cells, FABP4 was observed at lower levels than in normal prostate epithelial cells, and its upregulation enhanced apoptosis [43,44]. Additionally, hepatocellular carcinoma cells with upregulated FABP4 exhibited a significant reduction in cell proliferation [22]. All of these results indicated the different roles of FABP4 relying on the type of tumors. Our study identified an appreciable increase in FABP4 in colorectal cancer, and its depletion observably restrained cell growth and induced apoptosis. *In vivo* experiments further validated the suppressive effect of FABP4 knockdown on tumor growth in colorectal cancer.

Tumor carcinogenesis, including that of colorectal cancer, has been attributed to the involvement of CSCs. It has been suggested that tumor stemness could be a primary mechanism for colorectal cancer initiation, tumourigenesis, development, metastasis, and recurrence [15,45]. Various stemness markers, such as EPHA1, Oct4, Sox2, and ALDHA1, have been employed to investigate the stemness characteristics of CSCs [46–48]. In our study, we established that FABP4 depletion significantly attenuated the stemness of colorectal cancer, as corroborated by the diminished expression of stemness markers, including Sox2, Oct4, and ALDHA1 in colorectal cancer cells. Glycolysis has been shown to enhance tumor growth, metastasis, and development by increasing glucose uptake and lactate production and is thus considered a hallmark of tumor cells [49,50]. Glut1 has been identified as a diagnostic marker and potential treatment target in various tumors due to its role in promoting glucose uptake [51]. LDHA has also been recognized as a glycolysis marker [52]. A prior study revealed that lncRNA LINRIS facilitated glycolysis by stabilizing IGF2BP2, thereby promoting colorectal cancer progression [53]. Our results indicated that FABP4 downregulation in colorectal cancer cells decreased glucose uptake, ECAR, lactate production, and the ATP/ADP ratio. Additionally, the protein expression of Glut1 and LDHA was significantly diminished following FABP4 depletion. Moreover, ROS, produced by oxidative stress, have been identified as pivotal factors in tumor onset, progression, and recurrence. Consequently, we also investigated the effect of FABP4 on ROS levels in colorectal cancer cells. Our findings showed that FABP4 knockdown substantially increased ROS levels. These findings suggest that FABP4 depletion suppresses stemness and glycolysis, enhances ROS production in colorectal cancer cells, and consequently inhibits the progression of colorectal cancer.

Emerging research suggests that ROS can influence cellular processes such as metastasis, apoptosis, and growth by modulating various pathways and factors, including the PI3K/Akt pathway, nuclear factor kappa B, ERK and vascular endothelial growth factor [54]. The MAPK/ERK pathway can also activate mTOR. The ERK/mTOR signaling pathway is a well-studied intracellular pathway in tumor progression, and its abnormal activation has been detected in various cancers, including mucoepidermoid carcinoma [55] and cervical cancer [56]. We treated colorectal cancer cells with ROS scavenger NAC to understand whether increased ROS production influences ERK/mTOR pathway activation. Previous studies have shown that NAC inhibits ROS accumulation, deactivating the ERK/mTOR pathway [56]. Consistent with these findings, our study demonstrated that FABP4 depletion could activate the ERK/mTOR pathway, which was hindered after addition of NAC. This suggests that FABP4 depletion may enhance ROS production to activate the ERK/mTOR pathway. Furthermore, our data indicated that NAC increased cell proliferation, stemness, and glycolysis while reducing apoptosis. In doing so, it mitigated the effects of FABP4 knockdown on colorectal cancer development. This proves that increased ROS production could be a key mechanism in suppressing stemness, glycolysis and proliferation and promoting apoptosis via the FABP4 depletion-mediated ERK/mTOR pathway.

Conclusions

Overall, these findings strongly demonstrated ROS production was a main mechanism in stemness, proliferation, glycolysis, and apoptosis of colorectal cancer cells, through which, FABP4 downregulation acted as a suppressor in the development of colorectal cancer through modulating the ROS/ERK/mTOR pathway.

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

Author Contributions

YCG and ZRZ designed the research study. YYW, XW and JWM performed the research. YYW, MW and NL analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study received approval from the First Hospital of Hebei Medical University (approval No.: S00164). Before their involvement, all patients provided their informed consent in writing.

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Conflict of Interest

The authors declare no conflict of interest.

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