ENO1 deletion potentiates ferroptosis and decreases glycolysis in colorectal cancer cells via AKT/STAT3 signaling

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Abstract. Colorectal cancer (CRC) is one of the most prevailing and lethal forms of cancer globally. α-enolase (ENO1) has been well documented to be involved in the progression and drug resistance of CRC. The present study was designed to specify the role of ENO1 in major events during the process of CRC and to introduce its latent functional mechanism. ENO1 expression was determined by western blot analysis. Extracellular acidification rates were assessed using an XF96 extracellular flux analyzer. Glucose uptake, lactic acid production, total iron levels and ferroptosis-related markers were examined with corresponding kits. A dichlorodihydrofluorescein diacetate probe measured intracellular reactive oxygen species content. Western blotting detected the expression of glycolysis- and ferroptosis-related proteins. CCK-8 and EdU staining assays assessed cell proliferation. In the current study, ENO1 was highly expressed in CRC cells. Knockdown of ENO1 markedly reduced the glycolysis and accelerated the ferroptosis in CRC cells. Moreover, the inhibitory effects of WZB117, a specific inhibitor of glycolysis-related glucose transporter type 1, on CRC cell proliferation were further enhanced by ENO1 interference. In addition, silencing of ENO1 inactivated the AKT/STAT3 signaling. The AKT activator SC79 partially reversed the effects of ENO1 deficiency on the AKT/STAT3 signaling, glycolysis, proliferation as well as ferroptosis in CRC cells. In summary, inactivation of AKT/STAT3 signaling mediated by ENO1 inhibition might boost the ferroptosis and suppress the glycolysis in CRC cells.

Introduction

Colorectal cancer (CRC) is a recognized form of gastrointestinal malignancy that encompasses colon and rectal cancer (1). Globally, an estimated 1.8 million new cases of CRC occur annually, the incidence rate and mortality rate of which respectively account for 10 and 9.4% of all cancers (2,3). The outcome of patients with CRC remains negative due to metastasis and recurrence in spite of the great advances that have been witnessed in the therapeutic modalities of CRC including surgery, chemotherapy, radiotherapy, immunotherapy and targeted therapy (4-6). Moreover, epidemiological studies have consistently displayed the obvious interaction between the risk of CRC and other human diseases, such as inflammatory bowel disease, obesity and diabetes (7,8). Therefore, the poorly understood molecular mechanism of CRC needs to be further elucidated and the potential key molecular drivers remain to be developed.

Enolase (ENO) is a key glycolytic enzyme that is responsible for the transformation of 2-phosphoglycerate into phosphoenolpyruvate (9). In addition to its role as a glycolytic enzyme, α-enolase (ENO1), the most extensively studied isoform of ENO, is expressed on the cell surface of most tumors and has been shown to be an oncogenic factor in multiple cancers that depends on the modulation of a variety of biological events, such as glycolysis, angiogenesis, invasion and metastasis (10,11). In particular, interference with ENO1 has been shown to decrease glycolysis, cell growth, metastasis and chemotherapy resistance in CRC (12-14).

Mounting evidence has demonstrated that multiple biological signaling cascades are involved in the occurrence and development of CRC (15,16). The PI3K/AKT and STAT3 pathways are both classical signaling pathways implicated in the diverse phenotypes of tumor cells (17,18). Notably, it is increasingly reported that AKT/STAT3 signaling is dysregulated and activation of JAK2/STAT3 signaling may contribute to the malignant progression of CRC (19,20). In addition, Sun *et al* (21) clarified that ENO1 may act as a modulator of AKT signaling to participate in the process of gastric cancer.

The present study endeavored to explore whether ENO1 functioned in CRC via mediating AKT/STAT3 signaling.

Materials and methods

Cell culture and treatment. Fetal bovine serum (10%; FBS; BeNa Culture Collection) was added to Dulbecco's Modified Eagle's Medium-high glucose (DMEM-H; BeNa Culture Collection), F-12K medium (BeNa Culture Collection), L-15 medium (BeNa Culture Collection) and Roswell Park Memorial Institute (RPMI)-1640 medium (BeNa Culture

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Collection) at 37˚C with 5% CO_2 for the incubation of CRC cell lines (Caco2, LoVo, SW480 and HCT116) procured from BeNa Culture Collection, separately. Human intestinal epithelial cell line (HIEC-6) procured from Zhejiang Meisen Cell Technology Co., Ltd., was also cultured in DMEM-H with 10% FBS. In addition, HCT116 cells were stimulated by 80 μM WZB117 (Shanghai Topscience Co., Ltd.) for 24 h (22) or 5 μM SC79 (Shanghai Topscience Co., Ltd.) for 2 h (23).

Transfection of plasmids. Using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), the specific small interfering (si)RNAs for ENO1 (siRNA-ENO1-1/2) and the corresponding negative control (siRNA-NC) from Guangzhou Geneseed Biotech Co., Ltd., were transfected into HCT116 cells. Briefly, $2x10^5$ cells were seeded in a 6-well plate. When the cell fusion efficiency was 30-50%, 2 ml of a pre-prepared transfection complex containing 50 nM siRNA was added to each well. The cells were cultured at 37˚C with 5% CO_2 for 6 h and then the medium was changed to continue the culture. The cells were collected 48 h post-transfection for further experiments. The siRNA sequences used were as follows: siRNA-ENO1-1: 5'-GTG TCCCTTGCCGTCTGCAAAGC-3', siRNA-ENO1-2: 5'-ATCAATGGCGGTTCTCATGCTGG-3', siRNA-NC: 5'-TCGTTGGCACTTCGGGTCTGCTAG-3'.

Cell Counting Kit-8 (CCK-8). Transfected HCT116 cells in 96-well plates were exposed to 80 μM WZB117 or 5 μM SC79 after the adjustment of cell density to $5x10^3$ cells/well. Each well was cultivated for extra 2 h at 37˚C following addition of 10 μl CCK-8 solution (Selleck Chemicals). Cell activity was assessed by estimating OD450 nm value by a microplate reader (Huaan Magnech Bio-Tech Co., Ltd.).

5-Ethynyl-2'-deoxyuridine (EdU) staining. Cell proliferation was measured via the employment of iClick EdU Andy Fluor 488 Imaging Kit (Guangzhou FulenGen Co., Ltd.). In brief, the transfected HCT116 cells ($1x10^4$ cells/well) subjected to 96-well plates were exposed to WZB117 or SC79, prior to the addition of EdU (20 μM per well) for 2 h at 37˚C as per the manufacturer's instructions. Afterwards, cells were probed with iClick EdU reaction buffer and stained with Hoechst 33342 for 10 min at room temperature following 15 min of fixation with 3.7% paraformaldehyde and 20 min of permeabilization with 0.5% Triton X-100 at room temperature. Images were prepared for observation under a fluorescence microscope (Motic China Group Co., Ltd.).

Estimation of total iron level. Following centrifugation at 3,000 x g for 10 min at 4˚C, total iron level in the cell supernatants was examined with Iron test kit (cat. no. ST1020; Leagene; Beijing Regan Biotechnology Co., Ltd.). OD562 nm value was determined using a spectrophotometer (Shimadzu Corporation).

Evaluation of ferroptosis markers. Following the centrifugation of HCT116 cells at 10,000 x g for 10 min at 4˚C, glutathione (GSH) and malondialdehyde (MDA) levels were examined with GSH Assay Kit (cat. no. TO1036; Leagene; Beijing Regan Biotechnology Co., Ltd.) and MDA Assay Kit (cat. no. TO1011; Leagene; Beijing Regan Biotechnology Co., Ltd.). The absorbance value at 412 and 535 nm was read with a spectrophotometer (Shimadzu Corporation).

Detection of intracellular reactive oxygen species (ROS). Intracellular ROS was estimated using a Reactive Oxygen Species Assay Kit (cat. no. BES-BK2782B; Shanghai Bolson Biotechnology Co., Ltd.). After indicated transfection and treatment, HCT116 cells plated into 24-well plate ($3x10^3$ cells/well) were treated with 10 μM dichlorodihydrofluorescein diacetate (DCFH-DA) at 37˚C for 20 min in the dark. The cells were then centrifuged at 1,000 x g for 5 min at 4˚C. Subsequently, the supernatant was removed and the cell pellet was resuspended in PBS. ROS production was determined under a fluorescence microscope at 480 nm excitation/590 nm emission.

Analysis of extracellular acidification rates (ECAR). The Seahorse XF Glycolysis Stress Test Kit (Agilent Technologies, Inc.) was used for the ECAR assay. Briefly, $5x10^4$ HCT116 cells were plated on 96-well cell culture XF microplates (Agilent Technologies, Inc.) for 24 h at 37˚C, which was then replaced by the medium without glucose and pyruvate. 10 mM glucose, 1 μM oligomycin and 100 mM 2-deoxy-glucose (2-DG) were sequentially added to the cell medium, with two measurements after each treatment. The results were subjected to analysis with Seahorse Bioscience XF96 Extracellular Flux Analyzer (Agilent Technologies, Inc.).

Detection of glucose consumption and lactate level. HCT116 cells inoculated in 96-well plates at a density of $2x10^6$ cells/well at 37˚C received the indicated transfection and treatment. Using a L-Lactate Assay Kit (cat. no. ab65330; Abcam) and Glucose Colorimetric Assay Kit (cat. no. ab282922; Abcam), lactate production and glucose consumption were respectively determined and were normalized to total protein concentration quantified by the BCA method (Shanghai Rongsheng Biotech Co., Ltd.).

Western blotting. Following the homogenization of HCT116 cells in RIPA buffer (Fude Biological Technology, Co. Ltd.), the BCA method (Shanghai Rongsheng Biotech Co., Ltd.) was used to measure protein content. Subsequently, 12 μg of protein was fractionated on 12% SDS-PAGE and transferring onto the PVDF membranes. The membranes were then blocked with 5% BSA for 1 h at room temperature (Beijing Solarbio Science & Technology Co., Ltd.) and immunoblotted with primary antibodies including ENO1 (cat. no. ab227978; 1:1,000), glucose transporter type 1 (GLUT1; cat. no. ab115730; 1:100,000), hexokinase 2 (HK2; cat. no. ab209847; 1:1,000), pyruvate kinase M2 isoform (PKM2; cat. no. ab85555; 1:1,000), glutathione peroxidase 4 (GPX4; cat. no. ab125066; 1:1,000), ferritin heavy chain 1 (FTH1; cat. no. ab75972; 1:1,000), acyl-CoA synthetase long-chain family member 4 (ACSL4; cat. no. ab155282; 1:10,000), AKT (cat. no. ab179463; 1:10,000), phosphorylated (p-)AKT (cat. no. ab38449; 1:1,000), STAT3 (cat. no. ab109085; 1:10,000), p-STAT3 (cat. no. ab267373; 1:1,000) and β-actin (cat. no. ab213262; 1:1,000) from Abcam at 4˚C overnight, prior to being incubated with HRP-conjugated secondary antibody (ab6721; 1:2,000;

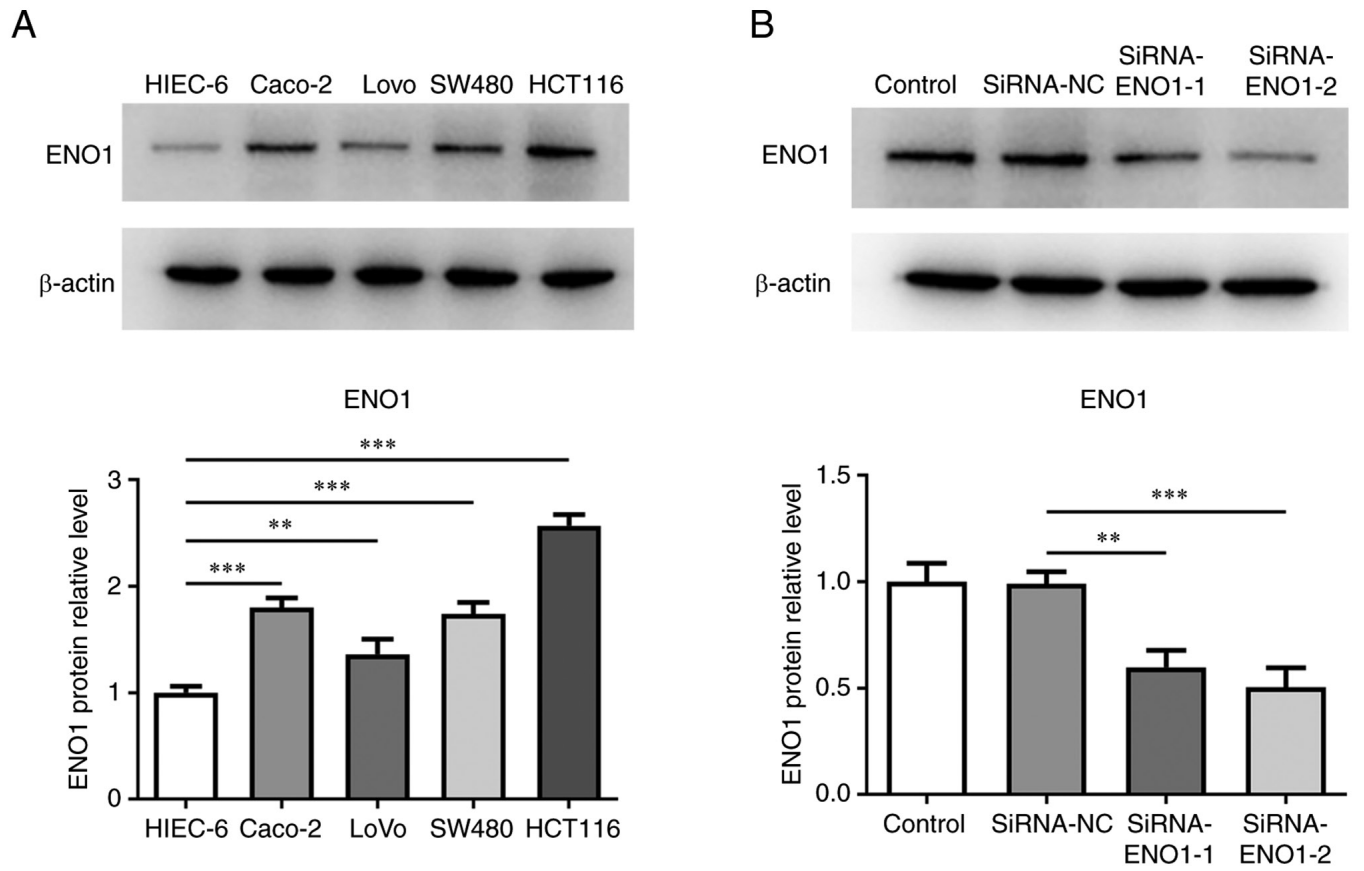

Figure 1. ENO1 expression is raised in CRC cells. (A) Western blotting of ENO1 expression. (B) Western blotting was used for the transfection efficiency of ENO1 interference plasmids. **P<0.01 and ***P<0.001. ENO1, α-enolase; CRC, colorectal cancer; SiRNA, short interfering RNA; NC, negative control.

Abcam) for 1.5 h at 37˚C. The visualization of the blots was performed by the Super ECL Plus from Biorigin (Beijing) Inc. and the densitometry was performed using ImageJ software (v. 1.4; National Institutes of Health).

Statistical analysis. Following analysis with GraphPad Prism 8 software (GraphPad Software; Dotmatics), all data were reported as mean ± standard error of mean. The unpaired student's t-test was used to compare the significance between two groups, and the one-way ANOVA and Tukey's post hoc test were used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

ENO1 expression is raised in CRC cells. To identify the role of ENO1 in CRC, ENO1 expression in CRC cells was initially tested. Using western blotting, it was found that ENO1 displayed higher expression in CRC cell lines (Caco2, LoVo, SW480 and HCT116) in contrast to a human intestinal epithelial cell line (HIEC-6; Fig. 1A). Accordingly, HCT116 cells were used in the following experiments. To determine the effects of ENO1 on the biological processes of CRC, ENO1 expression was distinctly weakened after transfection of siRNA-ENO1-1/2 plasmids. SiRNA-ENO1-2 was chosen for the follow-up assays since ENO1 showed lower expression in SiRNA-ENO1-2 group compared with the SiRNA-ENO1-1 group (Fig. 1B).

Deletion of ENO1 impedes the glycolysis of CRC cells. As depicted in Fig. 2A, following ENO1 knockdown, the ECAR was markedly decreased in HCT116 cells. In addition, inhibition of ENO1 resulted in the downregulation of glucose consumption and lactate production (Fig. 2B and C). Western blot analysis revealed that the expression of glycolysis-related GLUT1, HK2 and PKM2 were all lowered when ENO1 was depleted (Fig. 2D). All these findings suggested that ENO1 downregulation produced protective properties on the glycolysis of CRC cells.

ENO1 deficiency hampers the proliferation of CRC cells. As reported, glycolysis can serve as a driver of tumor cell proliferation. To investigate whether ENO1 participated in CRC cell proliferation via mediating glycolysis, WZB117, a specific inhibitor of GLUT1, was used. The experimental data from CCK-8 assay showed that the cell viability was reduced in the WZB117 group compared with the control group, and further attenuated after the simultaneous use of WZB117 and siRNA-ENO1 (Fig. 3A). As expected, EdU staining results showed that the fluorescence intensity of WZB117 group was slightly decreased compared with the control group, while after the use of SiRNA-ENO1, the fluorescence intensity of WZB117+iRNA-ENO1 group was significantly decreased compared with the other three groups (Fig. 3B). In conclusion, the suppressive role of GLUT1 inhibitor in CRC cell proliferation was strengthened by knockdown of ENO1.

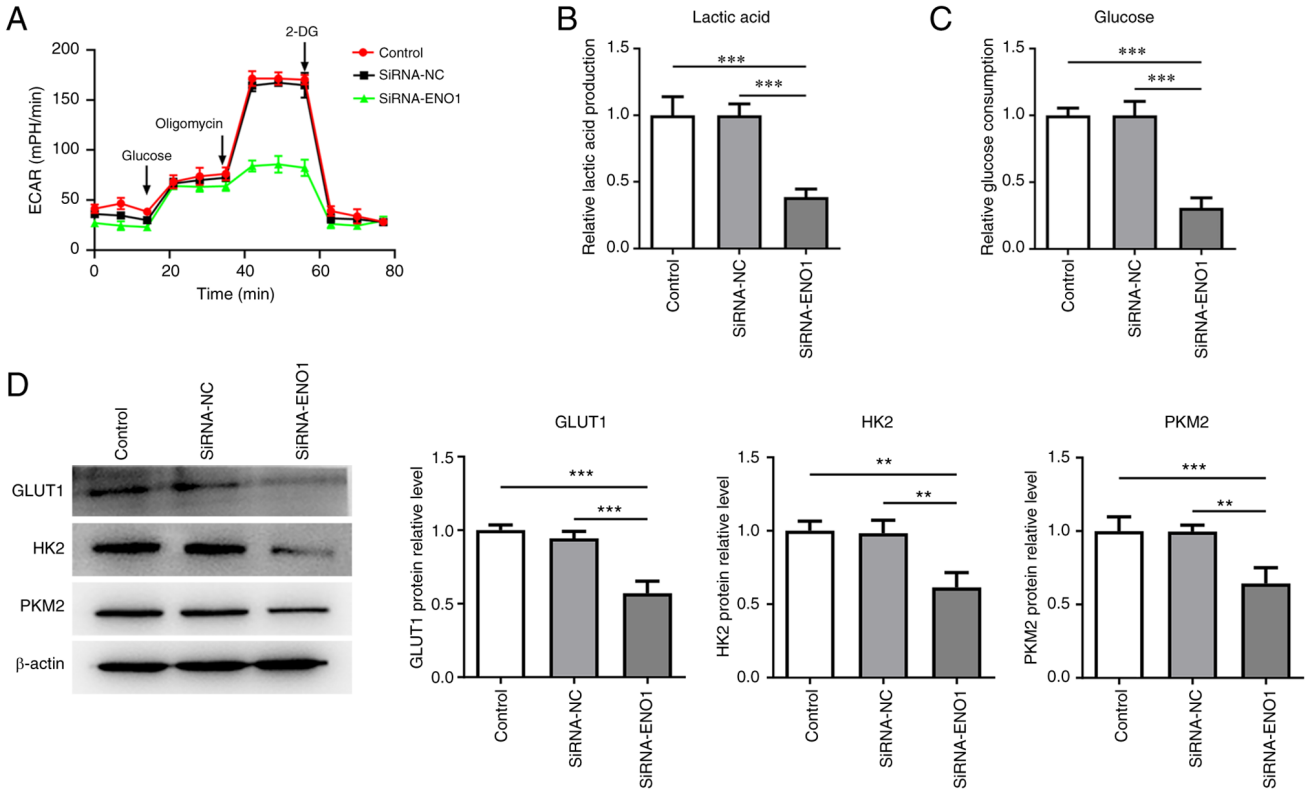

Figure 2. Deletion of ENO1 impedes the glycolysis of CRC cells. (A) Estimation of ECAR by XF96 extracellular flux analyzer. (B) Related kit assessed lactate production. (C) Related kit assessed glucose consumption. (D) Western blotting was used to examine the expression of glycolysis-related proteins. **P<0.01 and ***P<0.001. ENO1, α-enolase; CRC, colorectal cancer; ECAR, extracellular acidification rates; 2-DG, 100 mM 2-deoxy-glucose; SiRNA, short interfering RNA; NC, negative control; GLUT1, glucose transporter type 1; HK2, hexokinase 2; PKM2, pyruvate kinase M2 isoform.

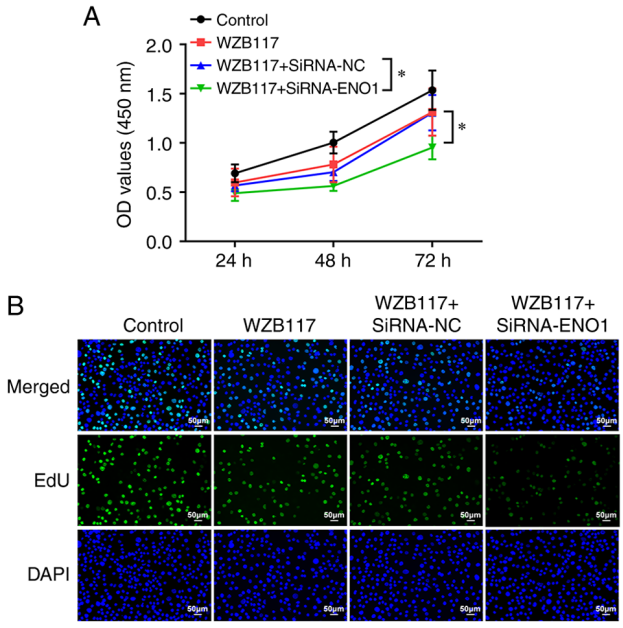

Figure 3. ENO1 deficiency hampers the proliferation of CRC cells. (A) CCK-8 method evaluated cell viability. (B) EdU staining assayed cell proliferation. *P<0.05. ENO1, α-enolase; CRC, colorectal cancer; EdU, 5-Ethynyl-2'-deoxyuridine; SiRNA, short interfering RNA; NC, negative control.

Absence of ENO1 intensifies the ferroptosis of CRC cells. Ferroptosis remains a pivotal event during the process of CRC (24). It was noted that ENO1 reduction evidently raised total iron and MDA levels but lessened GSH level (Fig. 4A and B). The results of DCFH-DA staining reflected that intracellular ROS level was noticeably elevated when ENO1 was downregulated (Fig. 4C). Western blotting also revealed that GPX4, FTH1 expressions were depleted and ACSL4 expression was augmented in ENO1-silenced HCT116 cells (Fig. 4D). Overall, ENO1 insufficiency might contribute to the ferroptosis of CRC cells.

Knockdown of ENO1 inactivates AKT/STAT3 signaling to decrease the glycolysis, proliferation and potentiate the ferroptosis of HCT116 cells. At the same time, the expressions of proteins involved in AKT/STAT3 signaling were examined and it proved that inhibition of ENO1 significantly depleted p-AKT/AKT and p-STAT3/STAT3 expressions, which were then both partially recovered by treatment with SC79, an activator of AKT (Fig. 5A), suggesting that ENO1 deletion blocked AKT/STAT3 signaling in CRC cells. Moreover, SC79 was further applied to show that ENO1 participated in the glycolysis and ferroptosis of CRC cells through mediating AKT/STAT3 signaling. SC79 was discovered to increase the ECAR which was on a downward trend in HCT116 cells transfected with SiRNA-ENO1 (Fig. 5B). In addition, the suppressed lactate production and glucose consumption caused by ENO1 interference were partially reversed by SC79 (Fig. 5C and D). GLUT1, HK2 and PKM2 expressions all fell in ENO1-silencing HCT116 cells, but were restored by SC79 (Fig. 5E). CCK-8 and EdU staining assays revealed that the attenuated proliferative capacity of HCT116 cells attributed to ENO1 insufficiency was

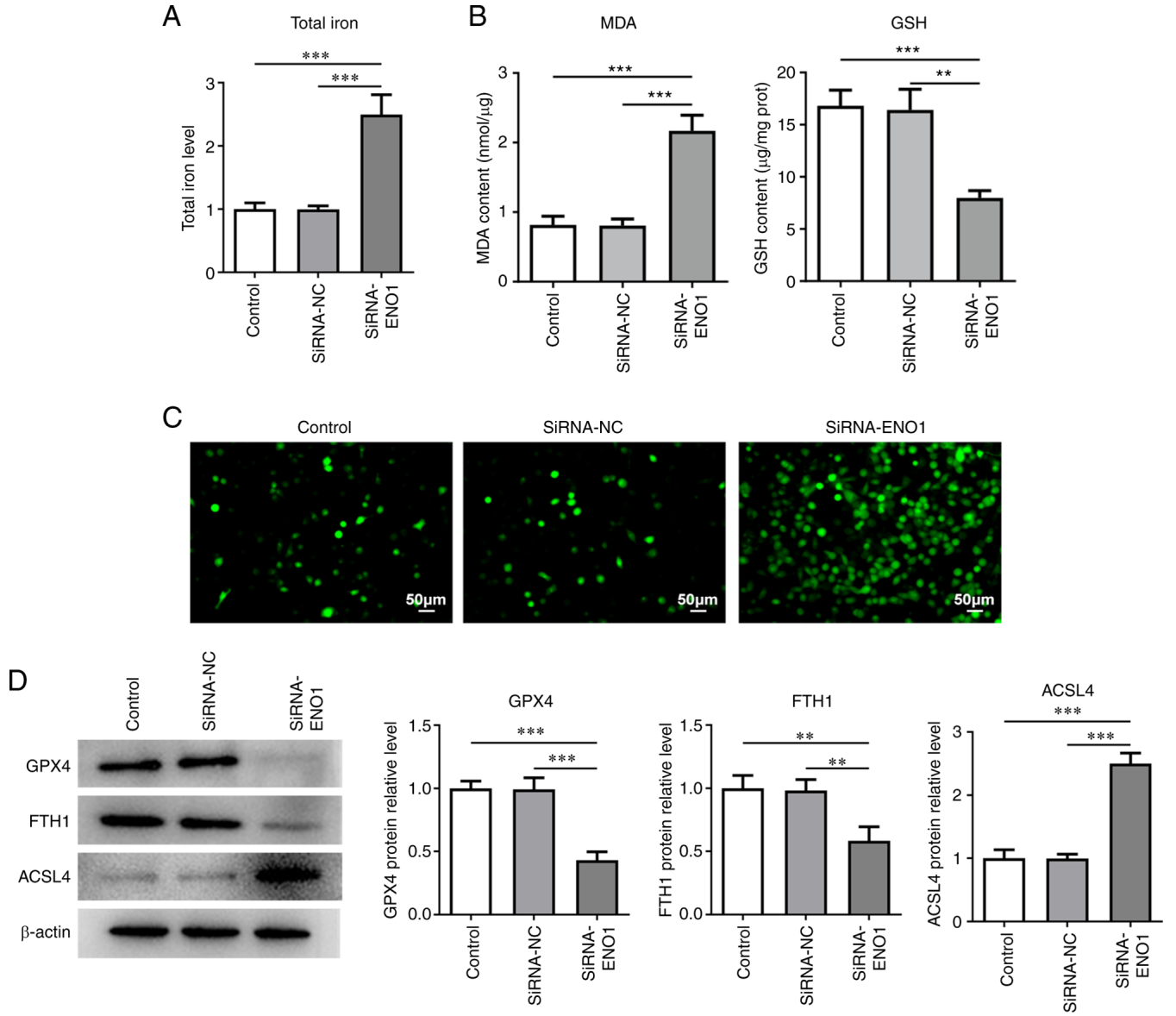

Figure 4. Absence of ENO1 intensifies the ferroptosis of CRC cells. (A) A related kit was used to measure total iron level. (B) Related kits were used to measure the contents of ferroptosis markers. (C) Dichlorodihydrofluorescein diacetate staining was used to estimate intracellular ROS activity. (D) Western blotting was used to examine the expression of ferroptosis-related proteins. **P<0.01 and ***P<0.001. ENO1, α-enolase; CRC, colorectal cancer; ROS, reactive oxygen species; SiRNA, short interfering RNA; NC, negative control; GPX4, glutathione peroxidase 4; FTH1, ferritin heavy chain 1; ACSL4, acyl-CoA synthetase long-chain family member 4.

improved by activation of AKT (Fig. 6A and B). Furthermore, the increasing total iron and MDA levels and the falling GSH level following knockdown of ENO1 in HCT116 cells were all reversed by SC79 (Fig. 7A and B). Similarly, absence of ENO1 accelerated the generation of intracellular ROS, which was halted by SC79 (Fig. 7C). ENO1 deletion also reduced GPX4 and FTH1 expressions while increasing ACSL4 expression, which were all nullified by SC79 (Fig. 7D). Activation of AKT/STAT3 signaling counteracted the effects of ENO1 deficiency on the glycolysis, proliferation and ferroptosis of CRC cells.

Discussion

CRC is a heterogeneous disease with different gene expression patterns and the identification of genetic molecular markers may be conducive to predict the prognosis of CRC and provide an alternative treatment option for CRC (25-27). ENO1 is a multifunctional protein that has been revealed to be aberrantly expressed in multiple human malignancies, such as breast cancer, gastric cancer, head and neck cancer, and to be related to the disease progression (10). Studies have also revealed that ENO1 expression is upregulated and expedites the tumorigenesis and metastasis in CRC (28,29). Consistent with these findings, ENO1 expression was notably elevated in CRC cells.

The oncogenesis and development of CRC is a multistep process. The dysregulation of metabolism is one of the most characteristic features of solid tumors and altered metabolic patterns are observed in various types of cancer cells (30). Specifically, cancer cells primarily absorb energy dependent on a metabolic phenotype known as aerobic glycolysis that is also recognized as a phenomenon termed the Warburg

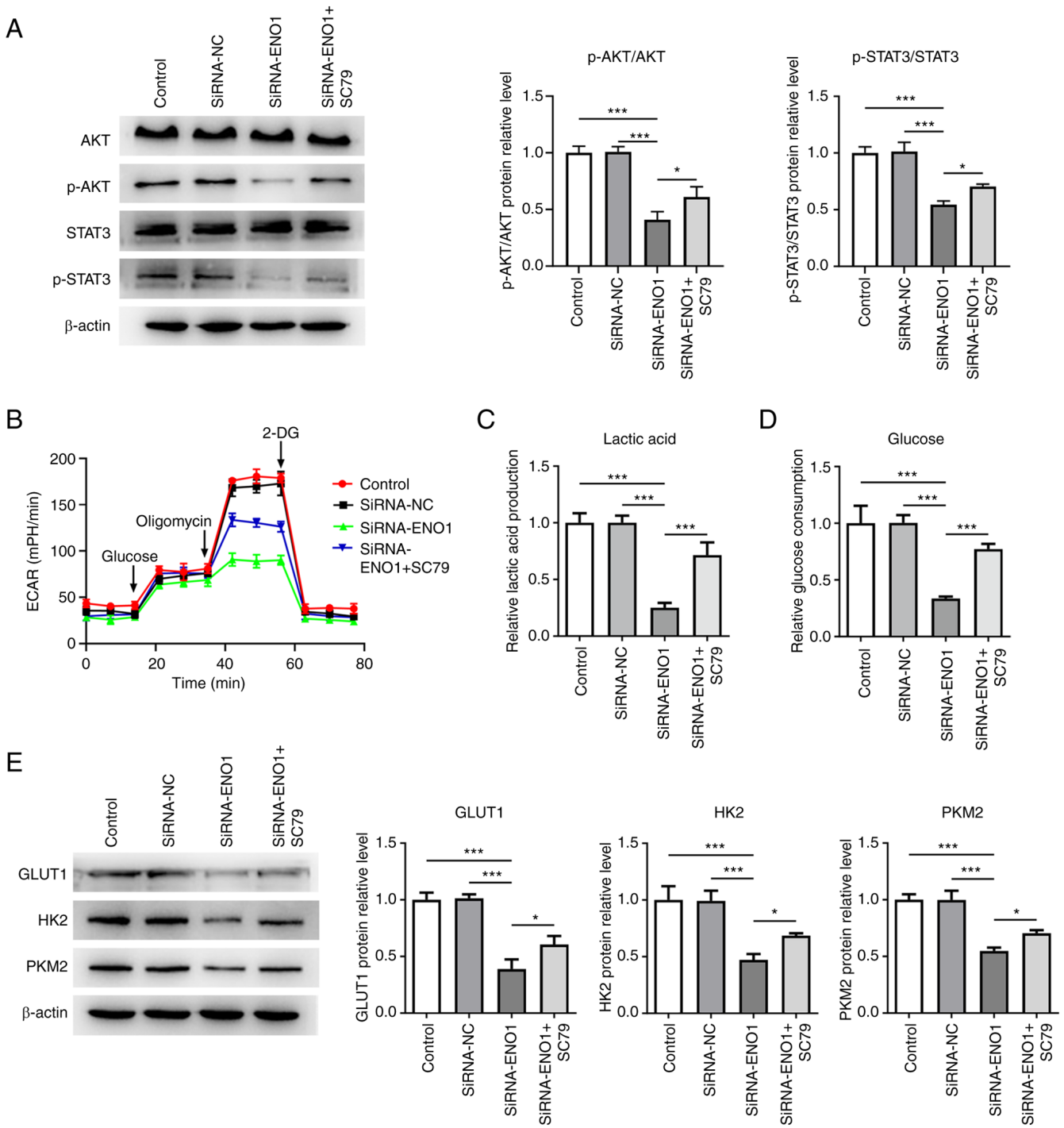

Figure 5. Knockdown of ENO1 inactivates AKT/STAT3 signaling to decrease the glycolysis of HCT116 cells. (A) Western blotting was used to examine the expression of proteins involved in AKT/STAT3 signaling. (B) Estimation of ECAR by XF96 extracellular flux analyzer. (C) A related kit was used to measure lactate production. (D) A related kit was used to measure glucose consumption. (E) Western blotting was used to examine the expression of glycolysis-related proteins. *P<0.05 and ***P<0.001. ENO1, α-enolase; CRC, colorectal cancer; ROS, reactive oxygen species; SiRNA, short interfering RNA; NC, negative control; p-, phosphorylated; 2-DG, 100 mM 2-deoxy-glucose; GPX4, glutathione peroxidase 4; FTH1, ferritin heavy chain 1; ACSL4, acyl-CoA synthetase long-chain family member 4.

effect, where cancer cells preferentially convert glucose to lactate (31). It is widely accepted that aerobic glycolysis plays a critical role in promoting the tumorigenesis of CRC (32,33). As an essential enzyme in the process of glycolysis, ENO1 has been hypothesized to exert pivotal functions in aerobic glycolysis in a variety of tumors, such as lung adenocarcinoma (34), pancreatic cancer (35) and gastric cancer (36). Zhan *et al* (14) verified that ENO1 induces lactate production in CRC cells. The present study also showed that ENO1 silencing might counteract glycolysis in CRC, as evidenced by reduced lactate production, glucose consumption and decreased ECAR in HCT116 cells. HK2 and PKM2 are identified as rate-limiting enzymes catalyzing the first or the final step of glycolysis pathway, respectively (37). GLUT1 is also a key rate-limiting factor in the transport and metabolism of glucose in cancer cells (38). In addition, aerobic glycolysis has

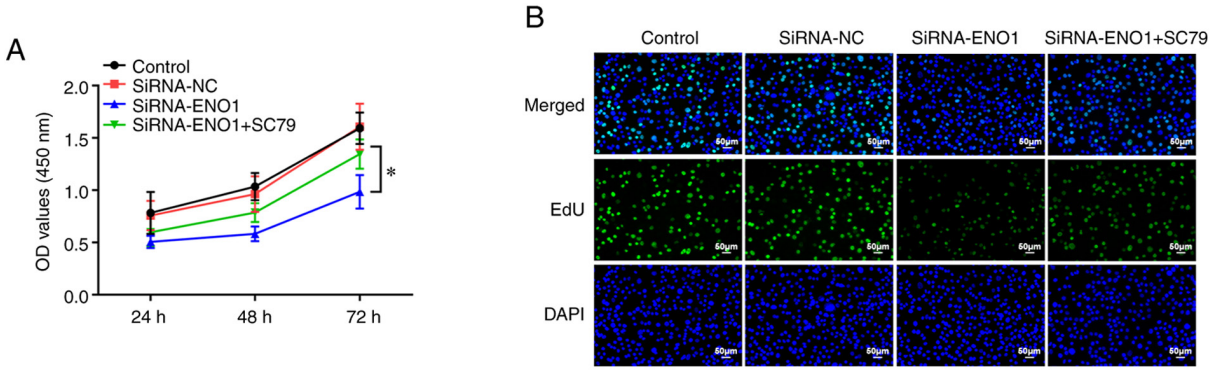

Figure 6. Knockdown of ENO1 inactivated AKT/STAT3 signaling to obstruct the proliferation of HCT116 cells. (A) CCK-8 method evaluated cell viability. (B) EdU staining assayed cell proliferation. *P<0.05. ENO1, α-enolase; EdU, 5-Ethynyl-2'-deoxyuridine; SiRNA, short interfering RNA; NC, negative control.

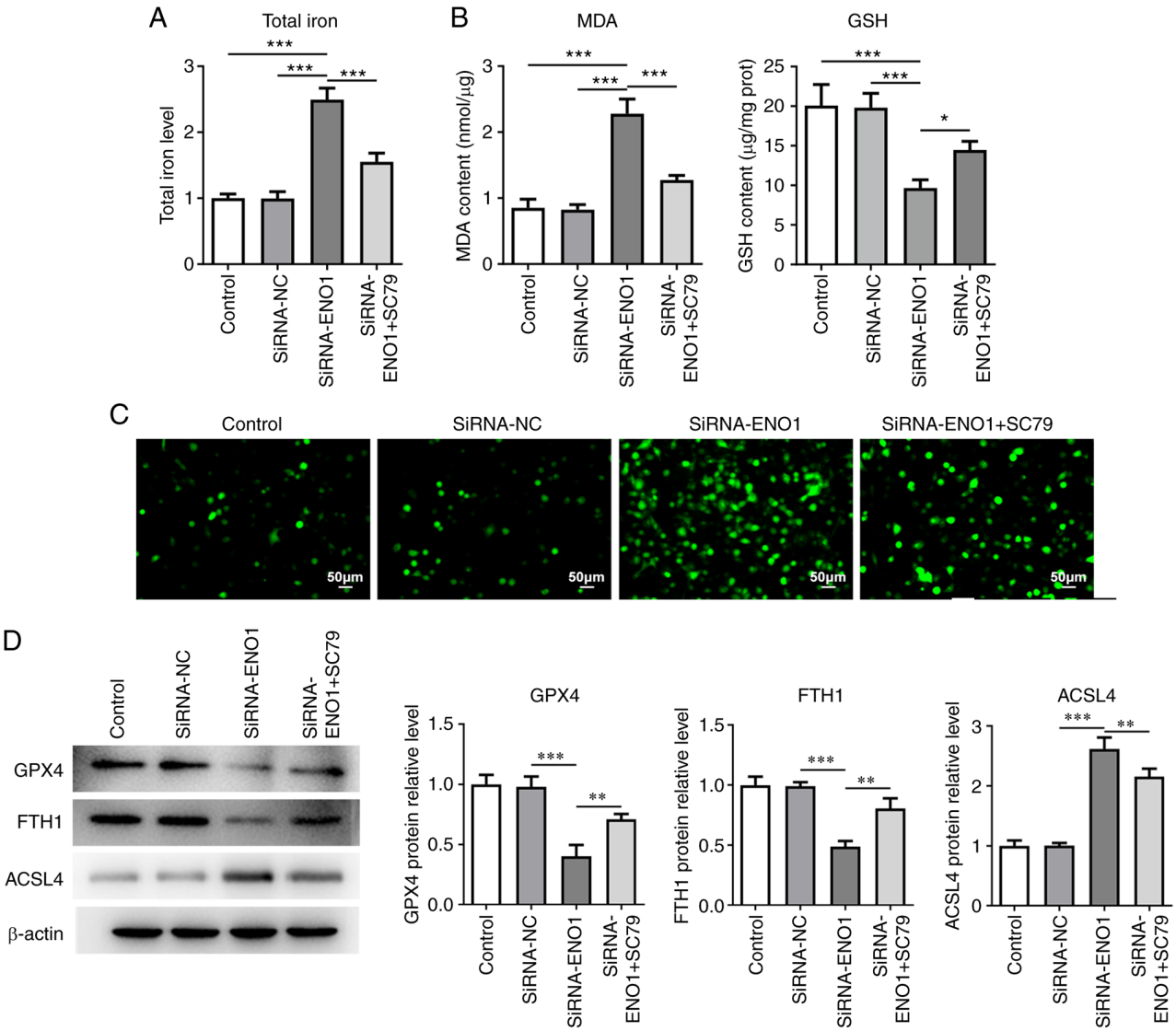

Figure 7. Knockdown of ENO1 inactivated AKT/STAT3 signaling to potentiate the ferroptosis of HCT116 cells. (A) A related kit was used to measure total iron level. (B) Related kits were used to measure the contents of ferroptosis markers. (C) DCFH-DA staining estimated intracellular ROS activity. (D) Western blotting was used to examine the expression of ferroptosis-related proteins. *P<0.05, **P<0.01 and ***P<0.001. ENO1, α-enolase; ROS, reactive oxygen species; SiRNA, short interfering RNA; NC, negative control; MDA, malondialdehyde; GSH, glutathione; GPX4, glutathione peroxidase 4; FTH1, ferritin heavy chain 1; ACSL4, acyl-CoA synthetase long-chain family member 4.

been reported to facilitate the proliferation of cancer cells (39). Meanwhile, ENO1 is considered to be a driver of CRC cell proliferation (14,29). As expected, in the present study, after ENO1 knockdown, GLUT1, HK2 and PKM2 expressions

all declined. The GLUT1 inhibitor WZB117 was used in the current study and it was noted that the viability and proliferation of CRC cells were both diminished by WZB117, which was further exacerbated by interference with ENO1.

Ferroptosis is a morphologically and biochemically novel form of cell death distinct from necrosis, autophagy, and apoptosis, and it is predominantly dictated by iron overload, lipid peroxidation and excess ROS production (40,41). Ferroptosis has been implicated in the etiology of CRC and targeting ferroptosis is a promising treatment strategy for CRC (42). Moreover, a recent study mentioned that ENO1 is highly associated with ferroptosis in hepatocellular carcinoma cells (43). GSH catalyzed by GPX4 is an intracellular antioxidant defense to scavenge the toxic lipid ROS and MDA is a product of lipid peroxidation (44,45). FTH1 is a ferroptosis-inhibiting protein while ACSL4 is a ferroptosis-promoting protein (46). ENO1 depletion was found to play a stimulatory role in ferroptosis events which were manifested as depleted GSH, GPX4 and FTH1, elevated iron release, ROS production, MDA and ACSL4.

AKT, belonging to the serine/threonine kinase family, is a proto-oncogene that can mediate cancer cell ferroptosis and glycolysis (47,48). STAT3 is an important transcription factor that also participates in the ferroptosis and glycolysis of tumor cells (49,50). Furthermore, AKT serves as an upstream regulator of STAT3 and may activate STAT3 (51). Concurrently, interference with ENO1 can reduce p-AKT expression in gastric cancer (21). In the present study, it was also observed that the lowered p-AKT/AKT and p-STAT3/STAT3 expressions in HCT116 cells caused by absence of ENO1 were then both partially enhanced by treatment with AKT activator SC79. Abundant evidence has demonstrated that the AKT/STAT3 signaling pathway plays a vital role in the ferroptosis and glycolysis in CRC (52-54). Furthermore, the present experimental results also validated that SC79 reversed the influences of ENO1 on the glycolysis, proliferation as well as ferroptosis of HCT116 cells.

Altogether, ENO1 might stimulate glycolysis and obstruct ferroptosis in CRC cells by regulating AKT/STAT3 signaling. This finding suggested ENO1 as a prospective target for CRC and implied that inhibition of ENO1 may provide and effective therapeutic strategy for CRC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YL performed most of the experiments and wrote the manuscript. YL and FZ were involved in cell culture. YH assisted YL in performing western blot analyses and FZ assisted YL in conducting CCK-8, EdU and ROS experiments. YL and YH both participated in the statistical analysis. YH and XW designed and supervised the study. YL and XW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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