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Inhibition of ubiquitin specific peptidase 8 is effective against 5-fluorouracil resistance in colon cancer via suppressing EGFR and EGFR-mediated signaling pathways

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Summary. Background. The identification of a sensitizing strategy to overcome 5-fluorouracil (5-FU) therapeutic resistance is needed in colon cancer. Recent studies highlight the oncogenic role of ubiquitin specific peptidase 8 (USP8) in many cancers. In line with these efforts, this work investigated the therapeutic potential of targeting USP8 in colon cancer.

Methods. Immunohistochemistry was performed to determine USP8 expression level in colon cancer tissues and their adjacent normal tissues. Gain-of-function analysis via plasmid overexpression and loss-of-function analysis via siRNA knockdown were applied on cellular assays. The combinatory effects of USP8 inhibitor and cisplatin were determined using a colon xenograft mouse model. Immunoblotting was performed to investigate the molecular mechanism of USP8 inhibition in colon cancer cells.

Results. Compared to normal counterparts, we showed that USP8 protein level was significantly higher in colon cancer tissues and cells. In addition, USP8 expression was not affected by prolonged exposure of colon cancer cells to 5-FU. USP8 was important for colon cancer cell growth and survival but not migration as assessed by loss-of-function and gain-of-function approaches. Pharmacological inhibition of USP8 using USP8 inhibitor is active against both sensitive and 5-FU-resistant colon cancer cells. Of note, USP8 inhibitor significantly inhibited colon cancer formation and growth, and augmented *in vivo* efficacy of 5-FU without causing toxicity in mice. Mechanistic studies showed that USP8 inhibitor acted on colon cancer cells through suppressing EGFR and EGFR-mediated signalling pathways.

Conclusions. Our work is the first to reveal the essential role of USP8 in colon cancer via EGFR oncogenic signalling pathways. Our findings provide a proof-of-concept that USP8 inhibitors are promising candidates to overcome 5-FU resistance in colon cancer.

Key words: USP8, EGFR, Therapeutic target, Colon cancer

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Introduction

Colorectal cancer (CRC) is the third most common cause of cancer mortality for men and women worldwide, and its incidence is rising rapidly (Bray et al., 2018). Of new colorectal cancer diagnoses, 20% of patients are accompanied by distant metastases, and approximately 40% who present with localized disease will later develop metastases. The median survival of metastasis CRC is only 6-12 months (Rumpold et al., 2020). Systemic chemotherapy is the primary treatment for metastatic CRC, but patients develop resistance and eventually relapse. Inactivation of tumor suppressor genes and mutation of oncogenes which lead to aberrant activation of downstream signalling pathways play essential roles in CRC occurrence, progression and chemoresistance (Nguyen and Duong, 2018). Recent clinical trials demonstrate that tailoring treatment to the molecular and pathologic features of the tumor improves overall survival (Biller and Schrag, 2021). Identifying molecules that are essentially involved in CRC is required to develop sensitizing strategy to overcome resistance.

Ubiquitin-specific proteases (USPs), the largest family of deubiquitinating enzymes which can remove ubiquitin from the substrate and protect the substrate from degradation, are emerging as therapeutic targets for human diseases related to aberrations in the ubiquitin-

proteasome system, such as cancer (Antao et al., 2020). Compared to other USPs, USP8 has garnered attention in recent years for cancer treatment because of its unique role in promoting cancer progression and resistance via stabilizing and activating numerous oncogenes and their downstream signaling pathways (Islam et al., 2021). USP8 regulates oncogenic receptor tyrosine kinases and tyrosine phosphatase such as CDC25, ERBB2 (Erb-B2 Receptor Tyrosine Kinase 2) and EGFR (epithelial growth factor receptor), which are all clinically relevant cancer targets. USP8 is highly expressed or hyper-activated in cancers derived from cervix, breast, lung and stomach, and is associated with poor prognosis (Baykara et al., 2013; Kim et al., 2017; Yan et al., 2018; Sun et al., 2020). USP8 inhibition suppresses growth, metastasis, and invasion, and induces death in several cancer models (Byun et al., 2013; Jeong, 2015; Jian et al., 2016).

In this study, we asked whether USP8 is a novel therapeutic target to overcome 5-FU resistance and what signaling pathways USP8 mediates in colon cancer. To address these questions, we firstly investigated expression pattern of USP8 in colon cancer tissues and cell lines. We next determined USP8 function in colon cancer growth, survival and migration. The effects of USP8 inhibitor alone and its combination with 5-FU were examined using both *in vitro* and *in vivo* colon cancer models. We finally analyzed the underlying mechanisms of USP8 inhibition focusing on EGFR-mediated signaling pathways.

Materials and methods

Cell culture, compounds and antibodies

Human colon cancer cell lines HCT-116, DLD-1, SW620 and Caco-2, and human colon normal epithelial cell line CRL-1831 were obtained from the American Type Culture Collection (ATCC, USA) and the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell line authentication was performed via human 9-Marker STR DNA profile analysis (Xpbiomed Inc.). Cell lines were cultured in Minimal Essential Media supplemented with 10% fetal bovine serum (FBS, Hyclone, UK), 2 mM L-glutamine (Invitrogen, USA) and 100 I.U./mL penicillin and 100 μg/mL streptomycin. Chemo-resistant HCT-116-r and SW620-r cell lines were established and maintained in the same medium as described in our previous study (Xi et al., 2018). Primary human colon epithelial cells (HCnEpC) were obtained from Cell Applications, Inc. and were cultured according to manufactures' recommendations. Fluorouracil (5-FU, Sigma) and USP8 inhibitor DUBs-IN-2 (Colombo et al., 2010) (MedChemExpress Inc.) were reconstituted in dimethyl sulfoxide (DMSO, Sigma), and were kept in -20°C as aliquot. Except anti-β actin antibody (Santa Cruz Biotechnology Inc.), all antibodies used in our study were obtained from Abcam Inc.

Patients, tissue specimen and immunohistochemistry

This study was approved and performed according to the guidelines by the ethics committee of Jingzhou Hospital. Sixteen paired colon cancer tissues and their adjacent normal tissues (at least 3 cm from the macroscopic tumor margin) were obtained from patients undergoing curative resection for colon cancer, with informed consent obtained from all patients. Tissue immunohistochemistry was performed using the standard protocol (Sakr et al., 2010). Briefly, the specimens were sectioned at a thickness of 4 μm and fixed with 4% paraformaldehyde, followed by staining with USP8 antibody (Abcam, ab228572), secondary antibody and DAB (3, 3-diaminobenzidine). Haematoxylin was applied as a counterstain. Quantification of staining was performed using Image J.

Cell proliferation and combination index

$5x10^3$ cells per well were seeded in 96-well plates for proliferation assay. Cells were treated with compounds for 3 days. After treatment, cell proliferation activity was determined using the BrdU proliferation assay kit (Promega) and absorbance was quantified on microplate reader (BioTek Instruments). The IC_{50} of single compound was determined using Prism 5. In combination studies, cells were treated with single compound alone and combination of two compounds using an equipotent constant-ratio concentration based on their IC_{50}. The CI at 0% to 100% growth inhibition was calculated using CalcuSyn software. CI>1.1 indicates antagonistic; CI at 0.9-1,1 indicates additive; and CI<0.9 indicates synergistic (Yadav et al., 2015).

Migration assay

Cell migration was measured by using the Boyden chamber with 8 μm pore sizes in 24-well transwell plates (Cell Biolabs Inc., USA). Cells together with compound in serum free medium were seeded in an insert placed in the upper chamber and complete medium with 10% serum was placed into lower chamber. After 8 hours incubation, unmigrated cells on upper side of insert membrane were removed. Migratory cells on lower sider of insert membrane were fixed with 5% glutaraldehyde and stained with 1% crystal violet. Migrated cells in five different random fields per well were counted under a light microscope.

Apoptosis assay

10^6 cells per well were seeded in 6-well plates for apoptosis assay. Cells were treated with compounds for 3 days. Cell apoptosis was assessed by using Cell Death Detection ELISA kit (Roche) via quantifying cytoplasmic histone-associated DNA fragments.

Absorbance was measured on microplate reader (BioTek Instruments).

Western blotting

Total proteins were extracted from 10^6 cells per sample using radioimmunoprecipitation assay (RIPA) buffer (Invitrogen). Protein concentration was determined using BCA method. Proteins at 50 µg were resolved by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (Bio-Rad). Western blotting was performed using standard protocol (Liu et al., 2014). All antibodies were purchased from Cell signaling Inc. Signals were developed using chemiluminescent substrates (Pierce) and quantified using Image J software.

Overexpression and knockdown

10^6 cells per well were seeded in 6-well plates. On reaching 80% confluence, cells were transfected with siRNA (300 nmol) or overexpression plasmid (2 µg) using Dharmafect Transfection Reagent (Dharmacon) as described in our previous study (Xi et al., 2018). siRNA against USP8: 5'-GGTTCAGGCAAGCCATTTA-3' and negative control siRNA were synthesized by RiboBio Inc. USP8 was cloned into pCMV3-C-his vector (Sino Biological). Protein expression analysis was performed at 48h post-transfection.

Transplantation of tumor cells into nude mice

All of the animal experiments were approved and performed according to institutional guidelines by Institutional Animal Care and Use Committee of Yangtze University. Colon xenograft mouse model was generated by subcutaneously implanting Caco-2 cells (10 million cells each) into SCID mice. To investigate the effects of compound on tumor formation, USP8 inhibitor was administrated to SCID mice via intraperitoneal injection on the same day of colon cancer cell inoculation. The percentages of tumor formation in each group were determined in the next two weeks of treatment duration. To investigate the effects of compound on tumor growth, compound was only given after the development of palpable tumor. Tumour length and width were measured every three days and tumor volume was estimated using formula: volume = length × $width^2/2$. When tumors reached approximately 100 mm^3, the mice were randomly divided into different groups (n=5 per group). Specific dose and duration of each compound are indicated in figure legends.

Statistical analyses

Data are expressed as mean and standard deviation to indicate data variability. Student's t test was performed by unpaired Student's t test for cell assays. A one-way analysis of variance (ANOVA) and the post-hoc Tukey honestly significant difference (HSD) test was used in the analysis. The determination of animal number follows that of previous similar work and are predictive of 80% power with alpha of 0.05 for type 1 error (Weiss et al., 2022). A p-value <0.05 was considered statistically significant.

Results

USP8 is highly expressed in colon cancer tissues and cells.

In order to investigate USP8 expression pattern in colon cancer, we performed immunohistochemistry using specific anti-USP8 antibody on colon cancer tissues and their adjacent normal tissues. Sixteen colon cancer patients were included in this study and their clinical characteristics are shown in Table S1. A representative immunohistochemical staining of USP8 is shown in Fig. 1A. We showed that USP8 expression was higher in colon cancer tissue than in the matching normal tissue. Quantification of USP8 staining using Image J showed that average of USP8 expression in colon cancer tissues was significantly increased by ~2-fold (Fig. 1B). We attempted to correlate USP8 expression level with patient clinical characteristics and found that USP8 upregulation was regardless of tumor stages, metastasis and histological differentiation (Table 1). In line with colon cancer tissues, we demonstrated that colon cancer cells had significantly higher levels of USP8 than normal colon epithelial cells (Fig. 1C,D). We further examined USP8 level in chemo-resistant HCT-116-r and SW620-r cells established in our previous study (Xi et al., 20218) to investigate whether USP8 level changes after prolonged exposure of chemotherapy. We did not detect any significant difference on USP8 level between parental and 5-FU-resistant colon cancer cell lines (Fig. 1E,F), suggesting that USP8 upregulation

Table 1. Clinicopathological information of colon cancer patients.

Patient	Age	Gender	Stage	Histological differentiation	Lymph node or distant Metastasis
P#1	≥60	Male	III	Well-moderate	Yes
P#2	<60	Female	I	Poorly/unknown	No
P#3	≥60	Male	III	Poorly/unknown	Yes
P#4	≥60	Male	III	Poorly/unknown	Yes
P#5	≥60	Male	II	Well-moderate	Yes
P#6	<60	Female	II	Well-moderate	No
P#7	≥60	Female	I	Well-moderate	No
P#8	≥60	Male	I	Poorly/unknown	No
P#9	≥60	Female	II	Well-moderate	No
P#10	<60	Male	II	Poorly/unknown	No
P#11	≥60	Male	III	Well-moderate	Yes
P#12	<60	Male	III	Well-moderate	Yes
P#13	≥60	Male	III	Poorly/unknown	Yes
P#14	≥60	Female	I	Well-moderate	No
P#15	<60	Male	I	Well-moderate	No
P#16	≥60	Female	III	Well-moderate	Yes

is unlikely to be involved in the development of chemoresistance.

USP8 is essential for colon cancer growth and survival.

In order to investigate the biological role of USP8 in colon cancer, we applied gain-of-function and loss-of-function approaches through plasmid overexpression and siRNA knockdown in a panel of colon cancer cell lines, including DLD-1, SW620, HCT-116 and Caco-2. We showed that cells transfected with USP8 overexpression plasmid displayed up to 4-fold increase in USP8 protein level compared to control cells (Fig. 2A). In addition, cells transfected with USP8 siRNA displayed a remarkable reduction in USP8 protein level compared to control cells (Fig. 3A). Results obtained from cellular assays demonstrated that USP8 overexpression significantly increased proliferation and decreased apoptosis-induced by serum reduction in all tested colon cancer cell lines (Fig. 2B,C). In contrast, USP8 knockdown significantly decreased proliferation and increased apoptosis (Fig. 3B,C). Of note, USP8 overexpression or inhibition did not affect colon cancer cell migration (Fig. 2D, 3D). These demonstrate that USP8 is important for colon cancer cell growth and survival but not migration.

USP8 inhibitor is effective in inhibiting colon cancer cells.

In order to investigate the therapeutic potential of

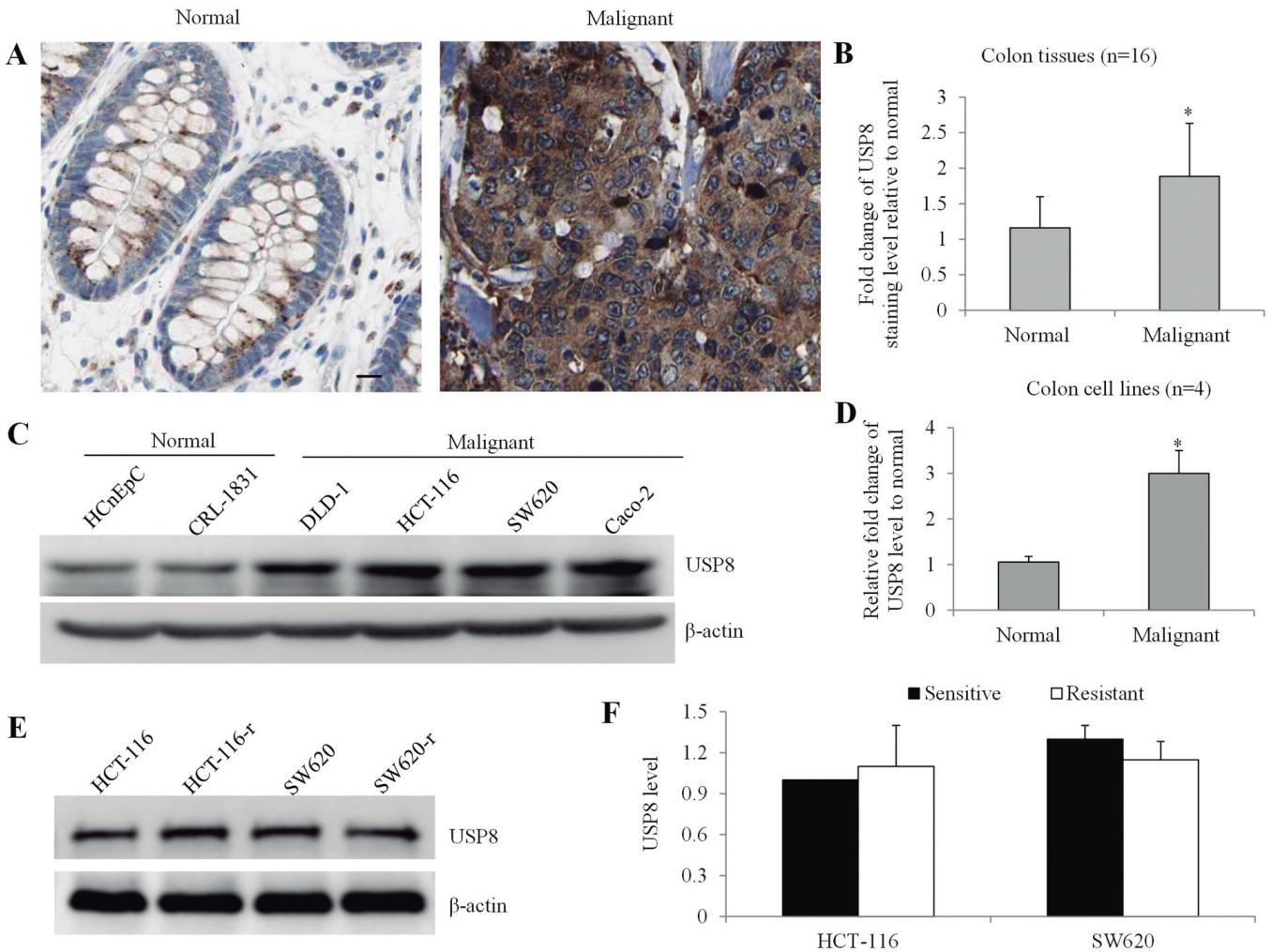

Fig. 1. USP8 is highly expressed in colon cancer tissues. **A.** Representative immunohistochemistry staining of USP8 in a pair of colon cancer and matching normal colon tissues. Haematoxylin stains cell nuclei blue. **B.** Average of USP8 on 16 pairs of colon cancer and matching normal colon tissues. Results are presented as relative to normal. Representative western blot image **(C)** and average level **(D)** of USP8 in human normal colon epithelial cells (HCnEpC and CRL-1831) and a panel of human colon cancer cell lines (DLD-1, HCT-116, SW620 and Caco-2). HCnEpC is primary human normal colon epithelial cells. CRL-1831 is an immortalized normal human colon epithelial cell line. Representative western blot image **(E)** and average level **(F)** of USP8 in chemo-sensitive and chemo-resistant HCT-116 and SW620 cells. *p<0.05, compared to normal. Scale bar: 5 μm.

targeting USP8 in colon cancer, we examined the efficacy of DUBs-IN-2, the specific USP8 inhibitor with an IC_{50} of 0.28 μM on USP8 activity and with IC_{50} >100 μM on other USPs (Colombo et al., 2010), on colon cancer cell proliferation, apoptosis and migration. We found that USP8 inhibitor at 1, 5 and 10 μmol/L significantly decreased proliferation and induced apoptosis in DLD-1 and Caco-2 cells in a dose-dependent manner (Fig. 4A,B). Consistent with USP8 knockdown, USP8 inhibitor did not affect colon cancer cell migration (Fig. 4C). We further challenged USP8 inhibitor on chemo-resistant cells and found that USP8 inhibitor was also effective in inhibiting proliferation and inducing apoptosis in HCT-116-r and SW62-r cells (Fig. 4D,E). USP8 inhibitor did not affect chemo-resistant cell migration (Fig. 4F).

USP8 inhibitor suppressed colon cancer formation and growth in mice.

In order to investigate the efficacy of USP8 inhibitor *in vivo*, we established a human xenograft colon cancer model through subcutaneously implanting Caco-2 cells in SCID mice. USP8 inhibitor at 1 mg/kg was administrated via intraperitoneal injection on the same day of tumor cell inoculation and was given to mice once per day for the next 14 days. We observed that the number of palpable tumors was significantly less in mice receiving USP8 inhibitor than control mice (Fig. 5A), demonstrating that USP8 inhibitor prevented colon cancer formation. We also treated mice with USP8 inhibitor after the development of palpable tumors (~ 150 mm^3) and monitored tumor growth curve of control and USP8 inhibitor treated-mice in the next three weeks. We found that USP8 inhibitor delayed colon cancer growth beginning at 6 days of the initial treatment and its inhibitory effect was observed throughout the duration of treatment (Fig. 5B). It was noted that there was no significant difference in mice body weight between control and USP8 inhibitor groups (Data not shown). In addition, we did not observe any abnormal appearance or behavior in mice receiving USP8 inhibitor. These suggest that the effective dose of USP8 inhibitor is not toxic to mice.

The combination of USP8 inhibitor and 5-FU is synergistic in colon cancer in vitro and in vivo.

Apart from USP8 inhibitor alone, we further investigated the combinatory effects of USP8 inhibitor

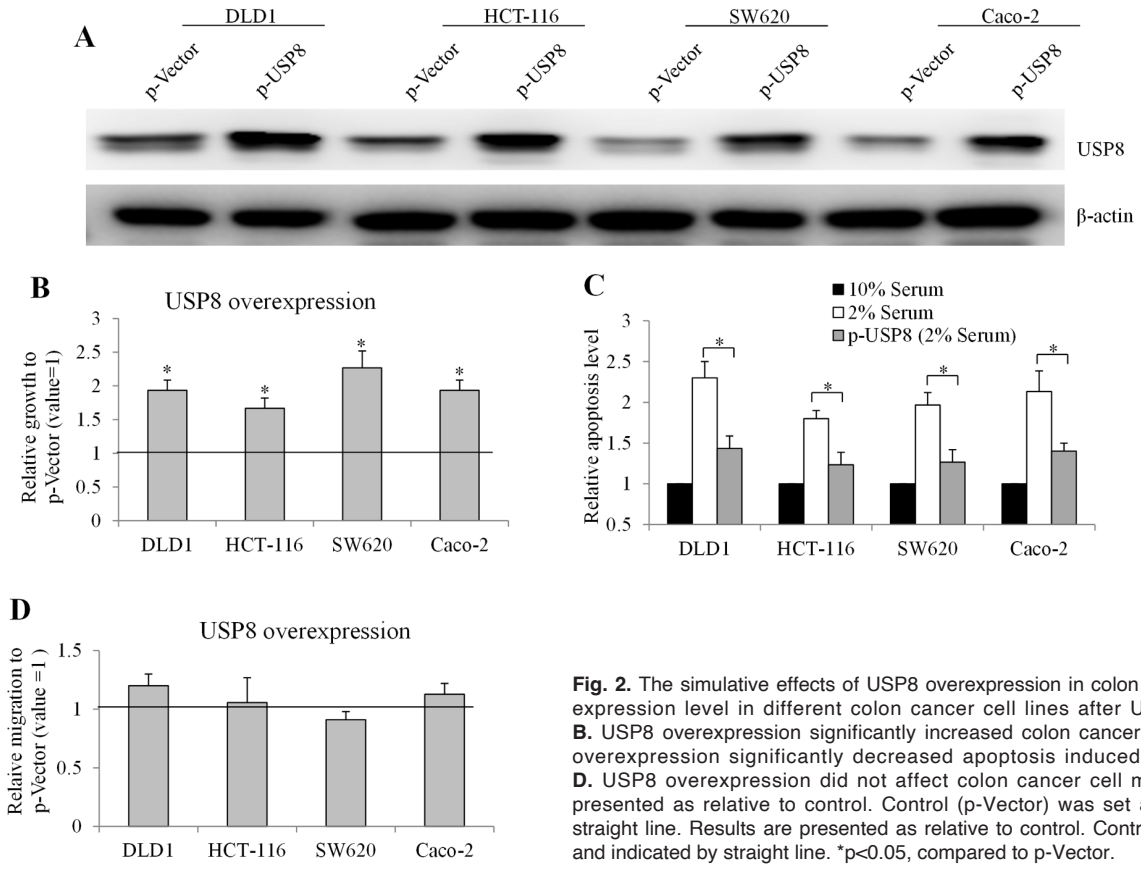

Fig. 2. The simulative effects of USP8 overexpression in colon cancer cells. **A.** USP8 expression level in different colon cancer cell lines after USP8 overexpression. **B.** USP8 overexpression significantly increased colon cancer cell growth. **C.** USP8 overexpression significantly decreased apoptosis induced by serum reduction. **D.** USP8 overexpression did not affect colon cancer cell migration. Results are presented as relative to control. Control (p-Vector) was set as 1 and indicated by straight line. Results are presented as relative to control. Control siRNA was set as 1 and indicated by straight line. *p<0.05, compared to p-Vector.

and 5-FU, the most common chemotherapeutic agent for colon cancer (Pardini et al., 2011). We designed combination studies based on the median-effect equation method (Chou et al., 2010). We treated colon cancer cells with increasing doses of USP8 inhibitor or 5-FU alone or an equipotent constant-ratio concentration of USP8 inhibitor and 5-FU, followed by proliferation measurement. Isobologram analysis showed that combination index of USP8 inhibitor and 5-FU were all less than 1.1 (Fig. 6A,B), demonstrating that the combination is synergistic in inhibiting colon cancer cell proliferation.

Using Caco-2 xenograft mouse model, while the USP8 inhibitor and 5-FU demonstrated effectiveness in inhibiting tumor growth as individual drugs, tumors in mice treated with a single agent still progressed to a similar size as the control group by day 50 (Fig. 6C). However, the combination of USP8 inhibitor and 5-FU resulted more effective in inhibiting tumor growth than administrating either agent alone. It is noted that we did not observe tumor progression until day 50. No overt signs of toxicity (such as weight loss, abnormal appearance and behavior) were observed in any treatment group during the experiment, which included mice treated with the drug combination.

USP8 inhibitor suppresses EGFR and EGFR-mediated signaling pathways in colon cancer cells.

USP8 has been shown to regulate levels of oncogenic receptor tyrosine kinase (RTK), such as EGFR (Byun et al., 2013; Jian et al., 2016). Pearson correlation coefficient analysis of mRNA expression of two genes in colon cancer tissues demonstrated that there was a positive medium correlation between EGFR and USP8 in colon cancer ($R = 0.45$, $P<0.001$) (Fig. 7A). Consistently, treatment of USP8 inhibitor at 5 and 10 μmol/L resulted in remarkable reduction of EGFR protein level (Fig. 7B,C). We observed the decreased p-EGFR at Y1173 and Y1068. As a consequence, phosphorylation of EGFR downstream effectors, including AKT and ERK1/2, were observed in colon cancer cells exposed to USP8 inhibitor. STAT3, an important signaling mediators downstream of activated EGFR (Gao et al., 2007), is activated by phosphorylation at T705 for nuclear translocation and DNA binding. We found that USP8 inhibitor significantly decreased p-STAT3 at T705, suggesting that USP8 inhibitor suppresses STAT3 activity. In addition, USP8 inhibitor significantly decreased STAT3 target gene c-Myc level in colon cancer cells (Fig. 7B,C). These results

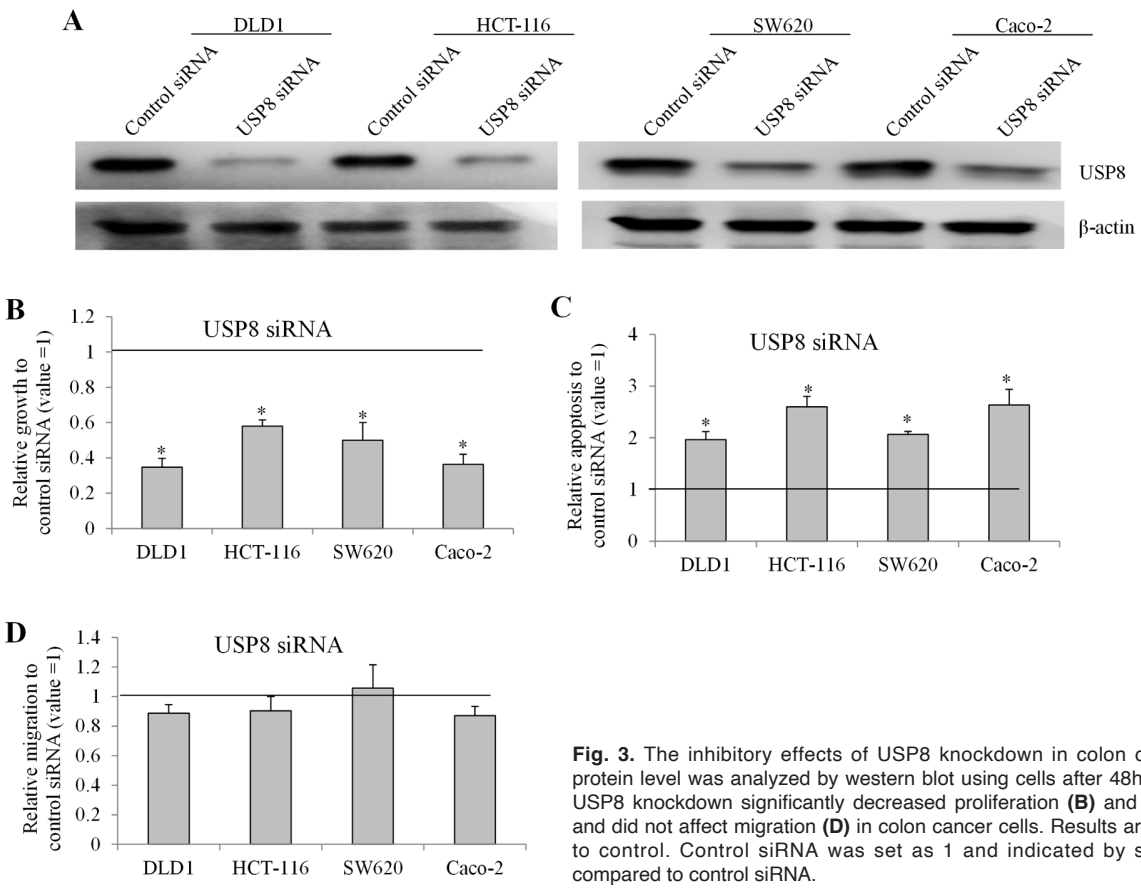

Fig. 3. The inhibitory effects of USP8 knockdown in colon cancer cells. **A.** USP8 protein level was analyzed by western blot using cells after 48h transfection of siRNA. USP8 knockdown significantly decreased proliferation **(B)** and induced apoptosis **(C)** and did not affect migration **(D)** in colon cancer cells. Results are presented as relative to control. Control siRNA was set as 1 and indicated by straight line. *$p<0.05$, compared to control siRNA.

demonstrate that USP8 inhibitor suppresses EGFR and EGFR-mediated signaling pathway in colon cancer cells.

Discussion

Recent clinical trials have demonstrated that tailoring treatment to the molecular and pathologic features of CRC improves overall survival (Biller and Schrag, 2021). With the advances in cancer genomics and proteomics, and the availability of patient-derived CRC models, substantial progress has been made in the development of targeted therapy which is emerging as a promising therapeutic strategy for the treatment of metastatic CRC. In line with these efforts, we previously identified that eIF4E (eukaryotic translation initiation factor 4E) contributes to colon cancer resistance, and furthermore that eIF4E is a druggable target (Xi et al., 2018). This work adds USP8 to the list of potential druggable targets that their inhibition is effective to sensitize colon cancer to chemotherapy.

Compared to normal colon tissue and cells, we showed that USP8 expression was increased in colon cancer tissue and cells. USP8 upregulation has also been observed in gastric cancer, lung adenocarcinoma and cervical cancer (Kim et al., 2017; Yan et al., 2018; Sun et al., 2020). Interesting, USP8 is downregulated in breast cancer (Qiu et al., 2018). Our work together with others demonstrate that USP8 overexpression is a feature in specific cancers. In addition, we did observe only some but not all colon cancer patients with USP8 overexpression, and this is not correlated with any clinicopathological characteristics, such as tumor stages, metastasis and histological differentiation. Pearson correlation coefficient analysis suggests a positive

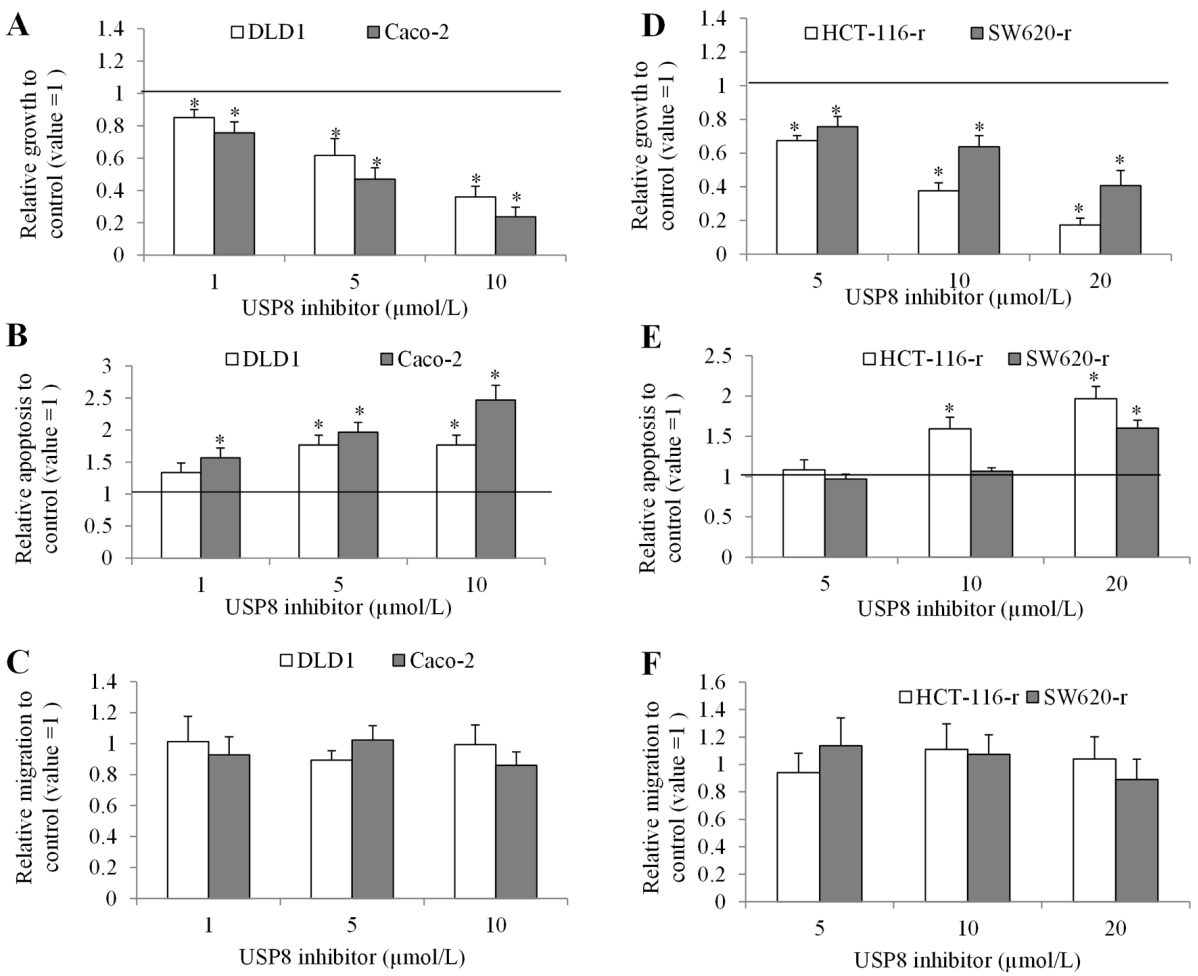

Fig. 4. USP8 inhibitor is active against chemo-sensitive and chemo-resistant colon cancer cells. USP8 inhibitor at 1, 5 and 10 μmol/L significantly inhibited proliferation **(A)** and induced apoptosis **(B)** of DLD-1 and Caco-2 cells. **C.** USP8 inhibitor did not affect DLD-1 and Caco-2 cell migration. USP8 inhibitor at 5, 10 and 20 μmol/L significantly inhibited proliferation **(D)** and induced apoptosis **(E)** of chemoresistant HCT-116-r and SW620-r cells. **F.** USP8 inhibitor did not affect DLD-1 and Caco-2 cell migration. Proliferation and apoptosis were analyzed after 3 days drug treatment. *p<0.05, compared to control.

medium correlation between EGFR and USP8 in colon cancer. This is in agreement with a previous finding that overexpression of EGFR was mutually correlated with that of USP8 in lung adenocarcinoma (Kim et al., 2017). The upregulation of USP8 expression and its correlation with clinicopathological characteristics should be confirmed in a larger cohort of colon cancer patients. The prognostic value of USP8 has been shown in lung, breast and gastric cancer (Baykara et al., 2013; Kim et al., 2017; Sun et al., 2020). It would be also important to investigate whether colon cancer patients with high USP8 level have poorer prognosis in a large cohort.

Using a combination of approaches, we showed that USP8 is important for colon cancer cell growth and survival. This finding is supported by others on the pro-proliferative and anti-apoptotic roles of USP8 in many cancers (Islam et al., 2021). Although USP8 has been reported to be involved in cancer cell migration (Rong et al., 2020; Sun et al., 2020), our findings showed that neither overexpression nor inhibition of USP8 affected colon cancer cell migration. We further show that specific USP8 inhibitor mimics anti-proliferative and pro-apoptotic activities of genetic knockdown of USP8. SW620 and HCT116 are colon cancer cell lines with high metastatic capacity. The effects of USP8 inhibitor on these two cell lines suggest that USP8 inhibition may be useful in patients with advanced metastatic conditions. We further showed that USP8 inhibitor at non-toxic dose effectively inhibited colon cancer

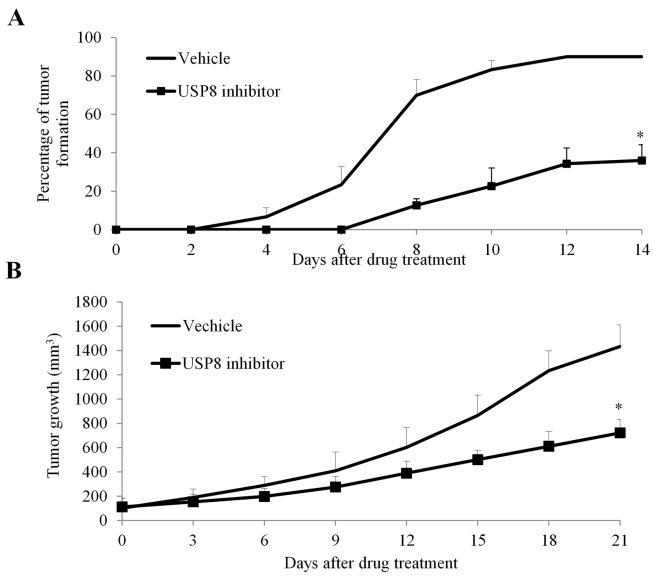

Fig. 5. USP8 inhibitor significantly prevented colon cancer formation and growth. **A.** Intraperitoneal injection of USP8 inhibitor (1 mg/kg) significantly decreased formation of colon cancer in mice. The percentage of tumor formation indicates the number of mice that formed tumor among total number of mice inoculated with tumor cells. Approximately 90% of mice with tumor inoculation formed tumor. Growth curve of tumors **(B)** from vehicle and 1 mg/kg USP8 inhibitor-treated mice. Tumor volumes were measured at the indicated time points as shown using the formula: Volume=(Length × Width^2) × 3.14/6 (n=5). *p<0.05, compared to control.

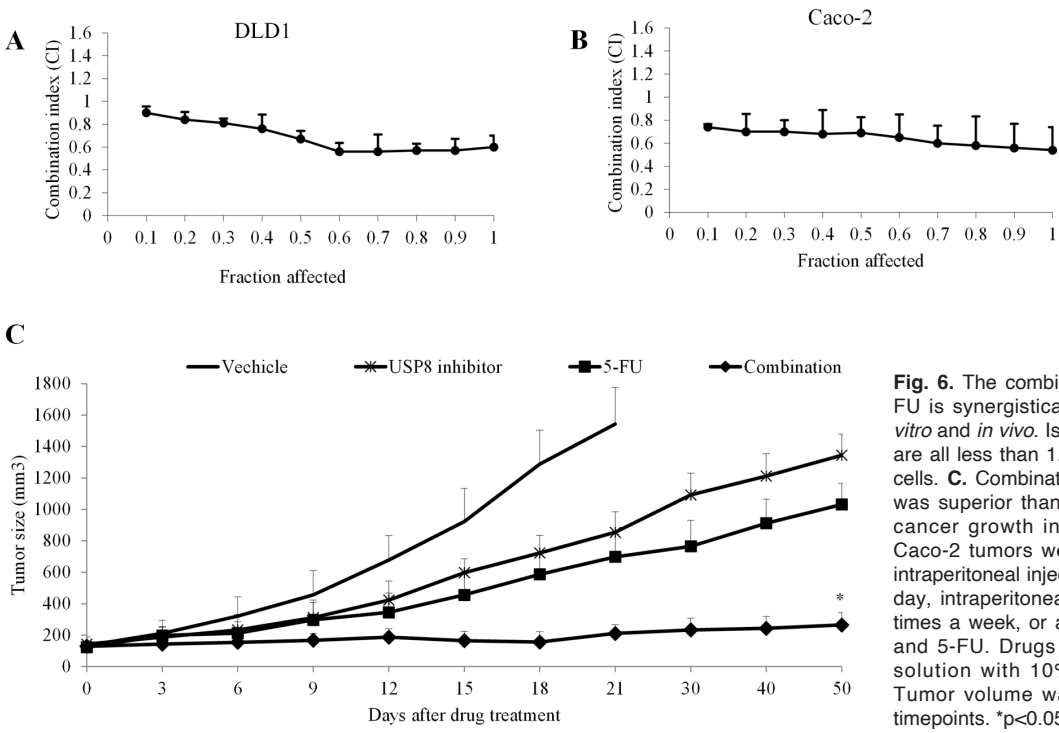

Fig. 6. The combination of USP8 inhibitor and 5-FU is synergistically effective in colon cancer *in vitro* and *in vivo*. Isobologram analysis of CI values are all less than 1.1 in DLD-1 **(A)** and SW620 **(B)** cells. **C.** Combination of USP8 inhibitor and 5-FU was superior than 5-FU alone in inhibiting colon cancer growth in mice. Mice with established Caco-2 tumors were treated with vehicle alone, intraperitoneal injection of 1 mg/kg USP8 once per day, intraperitoneal injection of 30 mg/kg 5-FU 3 times a week, or a combination of USP8 inhibitor and 5-FU. Drugs were dissolved in 0.9% NaCl solution with 10% Tween 80 and 1% DMSO. Tumor volume was measured at the indicated timepoints. *p<0.05, compared to 5-FU.

formation and growth in mice. This is significant as our work not only identifies a potential therapeutic target but also show that this target is druggable for colon cancer treatment. In addition, USP8 inhibitor was effective in inhibiting 5-FU-resistant colon cancer cells and acts synergistically with 5-FU *in vitro* and *in vivo*. The combination of USP8 inhibitor and 5-FU resulted in much better efficacy than 5-FU alone in inhibiting colon cancer growth in mice, and furthermore that no tumor progression was observed till an extension of growth period.

As a deubiquitinating enzyme, it is well known that USP8 maintains the ubiquitination level of several oncogenic receptor tyrosine kinases and regulates their downstream signaling pathways (Berruti and Paiardi, 2015; Smith et al., 2016; Kageyama et al., 2020). Our findings demonstrate that USP8 inhibitor acts on colon cancer cells via inhibiting EGFR signalling as both phosphorylation and total EGFR levels were remarkably reduced by USP8 inhibitor. As a consequence of EGFR inhibition, we observed the reduction of phosphorylation of EGFR downstream effectors by USP8 inhibitor. EGFR-targeted therapy using EGFR monoclonal antibody panitumumab in combination with chemotherapy has been shown to extend median survival by 2 to 4 months for patients with metastatic CRC (Douillard et al., 2014). Our work suggests that USP8 inhibitor is also a candidate to target EGFR in colon cancer. We found that USP8 inhibitor suppressed STAT3 activation and downstream signaling in colon cancer. STAT3 transcription factor is the target for anti-cancer therapy and STAT3 inhibitors have been used for the treatment of colon cancer (Mohassab et al., 2020). Our findings on the ability of USP8 inhibitor in suppressing STAT3 activation and overcoming chemoresistance in colon cancer cells are supported by recent work that STAT3 activation promotes colorectal cancer stem cell persistence and radio-resistance (Park et al., 2019).

In conclusion, our work links USP8 with EGFR in colon cancer and suggests that USP8 is important for colon cancer cell growth and survival but not migration. Thus, a combination of USP8 inhibitor and chemotherapy might present a promising strategy to eliminate resistant colon cancer cells.

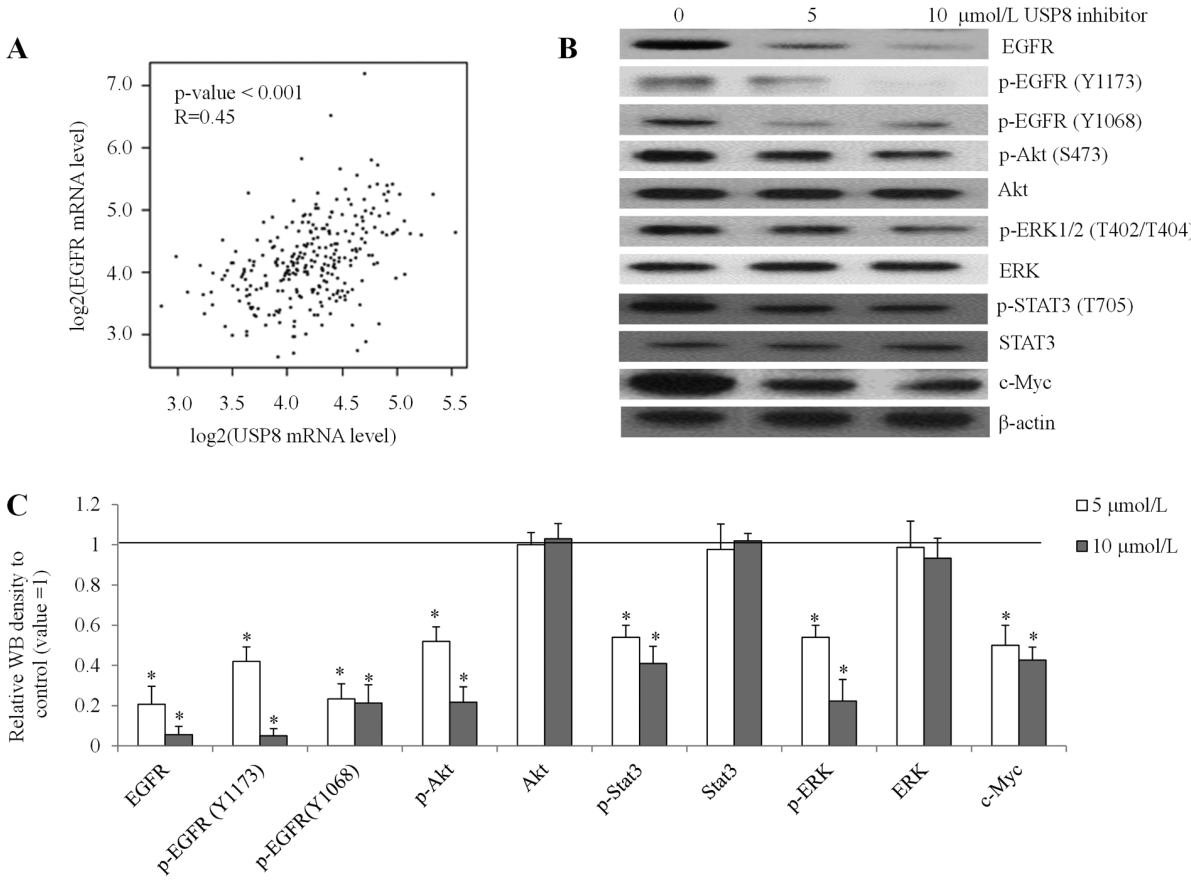

Fig. 7. USP8 inhibitor acts on colon cancer cells via inhibiting EGFR and EGFR-mediated signaling pathways. **A.** Pearson correlation coefficient analysis of EGFR and USP8 mRNA expression level in colon cancer tissues. TCGA database was used for this analysis. Representative western blot image **(B)** and quantification **(C)** of EGFR, p-EGFR, p-Akt, p-STAT3, p-ERK and c-Myc in Caco-2 cells after 24h USP8 inhibitor treatment. Results are presented as relative to control (0 μmol/L USP8 inhibitor). Control was set as 1 and indicated by straight line. *p<0.05, compared to control.

Acknowledgements. Not applicable.

Ethics approval and consent to participate. All the experimental protocols were approved by the Review Board of Jingzhou Hospital. Informed consent was obtained from all patients for sample collection. This study does not involve human participants. The procedures were carried out in accordance with the guidelines approved by the Ethics Committees of Jingzhou Hospital.

Consent for publication. Not applicable

Data Availability Statement. Data will be made available upon the reasonable request from corresponding author.

Competing interests. All authors declare no conflict of interest.

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Authors' contributions. CLX performed the experiments, analyzed the results and wrote the manuscript. JY supervised the project. ZLG, HY and LLC assisted the research and administrative work. All authors reviewed and approved the manuscript.

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