

# World Journal of *Gastroenterology*

*World J Gastroenterol* 2023 September 21; 29(35): 5094-5179



## REVIEW

- 5094 Developments and challenges in neoadjuvant therapy for locally advanced pancreatic cancer  
*Zhou B, Zhang SR, Chen G, Chen P*

## ORIGINAL ARTICLE

## Basic Study

- 5104 Regenerating gene 4 promotes chemoresistance of colorectal cancer by affecting lipid droplet synthesis and assembly  
*Zhang CY, Zhang R, Zhang L, Wang ZM, Sun HZ, Cui ZG, Zheng HC*

## Retrospective Cohort Study

- 5125 Clinical characteristics and outcome of autoimmune pancreatitis based on serum immunoglobulin G4 level: A single-center, retrospective cohort study  
*Zhou GZ, Zeng JQ, Wang L, Liu M, Meng K, Wang ZK, Zhang XL, Peng LH, Yan B, Pan F*

## Retrospective Study

- 5138 Machine learning-based decision tool for selecting patients with idiopathic acute pancreatitis for endosonography to exclude a biliary aetiology  
*Sirtl S, Żorniak M, Hohmann E, Beyer G, Dibos M, Wandel A, Phillip V, Ammer-Herrmenau C, Neesse A, Schulz C, Schirra J, Mayerle J, Mahajan UM*

## Clinical Trials Study

- 5154 Heparanase inhibition leads to improvement in patients with acute gastrointestinal injuries induced by sepsis  
*Chen TT, Lv JJ, Chen L, Li M, Liu LP*

## Observational Study

- 5166 Lowering the threshold of alanine aminotransferase for enhanced identification of significant hepatic injury in chronic hepatitis B patients  
*Yu HS, Jiang H, Li MK, Yang BL, Smayl A, Chen JN, Wu B, Yang YD*

## CORRECTION

- 5178 Correction to "Role of prebiotics, probiotics, and synbiotics in management of inflammatory bowel disease: Current perspectives"  
*Roy S, Dhaneshwar S*

**ABOUT COVER**

Editorial Board Member of *World Journal of Gastroenterology*, Konstantinos Triantafyllou, MD, PhD, FEBGH, Full Professor, Hepatogastroenterology Unit, Second Department of Internal Medicine-Propaedeutic, Medical School, National and Kapodistrian University of Athens, "Attikon" University General Hospital, Athens 12462, Greece. [ktriant@med.uoa.gr](mailto:ktriant@med.uoa.gr)

**AIMS AND SCOPE**

The primary aim of *World Journal of Gastroenterology* (WJG, *World J Gastroenterol*) is to provide scholars and readers from various fields of gastroenterology and hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. WJG mainly publishes articles reporting research results and findings obtained in the field of gastroenterology and hepatology and covering a wide range of topics including gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, gastrointestinal oncology, and pediatric gastroenterology.

**INDEXING/ABSTRACTING**

The WJG is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports, Index Medicus, MEDLINE, PubMed, PubMed Central, Scopus, Reference Citation Analysis, China Science and Technology Journal Database, and Superstar Journals Database. The 2023 edition of Journal Citation Reports® cites the 2022 impact factor (IF) for WJG as 4.3; Quartile category: Q2. The WJG's CiteScore for 2021 is 8.3.

**RESPONSIBLE EDITORS FOR THIS ISSUE**

Production Editor: *Yi-Xuan Cai*, Production Department Director: *Xiang Li*, Editorial Office Director: *Jia-Ru Fan*.

**NAME OF JOURNAL**

*World Journal of Gastroenterology*

**ISSN**

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

**LAUNCH DATE**

October 1, 1995

**FREQUENCY**

Weekly

**EDITORS-IN-CHIEF**

Andrzej S Tarnawski

**EXECUTIVE ASSOCIATE EDITORS-IN-CHIEF**

Xian-Jun Yu (Pancreatic Oncology), Jian-Gao Fan (Chronic Liver Disease), Hou-Bao Liu (Biliary Tract Disease), Naohisa Yoshida (Gastrointestinal Endoscopy)

**EDITORIAL BOARD MEMBERS**

<http://www.wjgnet.com/1007-9327/editorialboard.htm>

**PUBLICATION DATE**

September 21, 2023

**COPYRIGHT**

© 2023 Baishideng Publishing Group Inc

**PUBLISHING PARTNER**

Pancreatic Cancer Institute, Fudan University

Biliary Tract Disease Institute, Fudan University

**INSTRUCTIONS TO AUTHORS**

<https://www.wjgnet.com/bpg/gerinfo/204>

**GUIDELINES FOR ETHICS DOCUMENTS**

<https://www.wjgnet.com/bpg/GerInfo/287>

**GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH**

<https://www.wjgnet.com/bpg/gerinfo/240>

**PUBLICATION ETHICS**

<https://www.wjgnet.com/bpg/GerInfo/288>

**PUBLICATION MISCONDUCT**

<https://www.wjgnet.com/bpg/gerinfo/208>

**POLICY OF CO-AUTHORS**

<WebSite><https://www.wjgnet.com/bpg/GerInfo/310></WebSite>

**ARTICLE PROCESSING CHARGE**

<https://www.wjgnet.com/bpg/gerinfo/242>

**STEPS FOR SUBMITTING MANUSCRIPTS**

<https://www.wjgnet.com/bpg/GerInfo/239>

**ONLINE SUBMISSION**

<https://www.f6publishing.com>

**PUBLISHING PARTNER's OFFICIAL WEBSITE**

<WebSite><https://www.shca.org.cn/></WebSite><NewLine/>

<WebSite><https://www.zs-hospital.sh.cn/></WebSite>







## Basic Study

# Regenerating gene 4 promotes chemoresistance of colorectal cancer by affecting lipid droplet synthesis and assembly

Cong-Yu Zhang, Rui Zhang, Li Zhang, Zi-Mo Wang, Hong-Zhi Sun, Zheng-Guo Cui, Hua-Chuan Zheng

**Specialty type:** Gastroenterology and hepatology

**Provenance and peer review:** Unsolicited article; Externally peer reviewed.

**Peer-review model:** Single blind

**Peer-review report's scientific quality classification**

Grade A (Excellent): A  
Grade B (Very good): B  
Grade C (Good): 0  
Grade D (Fair): 0  
Grade E (Poor): 0

**P-Reviewer:** Batyrbekov K, Kazakhstan; Osera S, Japan

**Received:** June 25, 2023

**Peer-review started:** June 25, 2023

**First decision:** August 8, 2023

**Revised:** August 10, 2023

**Accepted:** August 25, 2023

**Article in press:** August 25, 2023

**Published online:** September 21, 2023



**Cong-Yu Zhang, Zi-Mo Wang, Hong-Zhi Sun, Hua-Chuan Zheng,** Cancer Center, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou 121001, Liaoning Province, China

**Rui Zhang,** Department of Colorectal Surgery, Liaoning Cancer Hospital, Shenyang 110042, Liaoning Province, China

**Li Zhang,** Department of Oncology, The Affiliated Hospital of Chengde Medical University, Chengde 067000, Hebei Province, China

**Zheng-Guo Cui,** Department of Environmental Health, University of Fukui School of Medical Sciences, Fukui 910-1193, Japan

**Corresponding author:** Hua-Chuan Zheng, MD, PhD, Professor, Cancer Center, The First Affiliated Hospital of Jinzhou Medical University, No. 2 Fifth Duan, Renmin Street, Guta District, Jinzhou 121001, Liaoning Province, China. [zheng\\_huachuan@hotmail.com](mailto:zheng_huachuan@hotmail.com)

## Abstract

### BACKGROUND

Regenerating gene 4 (*REG4*) has been proved to be carcinogenic in some cancers, but its manifestation and possible carcinogenic mechanisms in colorectal cancer (CRC) have not yet been elucidated. Our previous study found that the drug resistance of CRC cells may be closely linked to their fat metabolism.

### AIM

To explore the role of *REG4* in CRC and its association with lipid droplet formation and chemoresistance.

### METHODS

We conducted a meta-analysis and bioinformatics and pathological analyses of *REG4* expression in CRC. The effects of *REG4* on the phenotypes and related protein expression were also investigated in CRC cells. We detected the impacts of *REG4* on the chemoresistance and lipid droplet formation in CRC cells. Finally, we analyzed how *REG4* regulated the transcription and proteasomal degradation of lipogenic enzymes in CRC cells.

### RESULTS

Compared to normal mucosa, *REG4* mRNA expression was high in CRC ( $P < 0.05$ ) but protein expression was low. An inverse correlation existed between lymph

node and distant metastases, tumor-node-metastasis staging or short overall survival and *REG4* mRNA overexpression ( $P < 0.05$ ), but vice versa for *REG4* protein expression. *REG4*-related genes included: Chemokine activity; taste receptors; protein-DNA and DNA packing complexes; nucleosomes and chromatin; generation of second messenger molecules; programmed cell death signals; epigenetic regulation and DNA methylation; transcription repression and activation by DNA binding; insulin signaling pathway; sugar metabolism and transfer; and neurotransmitter receptors ( $P < 0.05$ ). *REG4* exposure or overexpression promoted proliferation, antiapoptosis, migration, and invasion of DLD-1 cells in an autocrine or paracrine manner by activating the epidermal growth factor receptor-phosphoinositide 3-kinase-Akt-nuclear factor- $\kappa$ B pathway. *REG4* was involved in chemoresistance not through de novo lipogenesis, but lipid droplet assembly. *REG4* inhibited the transcription of acetyl-CoA carboxylase 1 (ACC1) and ATP-citrate lyase (ACLY) by disassociating the complex formation of anti-acetyl (AC)-acetyl-histone 3-AC-histone 4-inhibitor of growth protein-5-si histone deacetylase;sterol-regulatory element binding protein 1 in their promoters and induced proteasomal degradation of ACC1 or ACLY.

## CONCLUSION

*REG4* may be involved in chemoresistance through lipid droplet assembly. *REG4* reduces expression of de novo lipid synthesis key enzymes by inhibiting transcription and promoting ubiquitination-mediated proteasomal degradation.

**Key Words:** Colorectal cancer; Regenerating gene 4; Aggressive behavior; Prognosis; Chemoresistance; Lipid droplet formation; Epidermal growth factor receptor signal

©The Author(s) 2023. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core Tip:** The roles of regenerating gene 4 (*REG4*) in colorectal cancer (CRC) and the related molecular mechanisms are still unknown. *REG4* may be involved in tumorigenesis and aggressiveness of CRC *via* the epidermal growth factor receptor-phosphoinositide 3-kinase-Akt-nuclear factor- $\kappa$ B pathway, and chemoresistance through lipid droplet assembly. *REG4* might be used as a useful marker for colorectal carcinogenesis and subsequent progression, as well as a potential gene therapy target.

**Citation:** Zhang CY, Zhang R, Zhang L, Wang ZM, Sun HZ, Cui ZG, Zheng HC. Regenerating gene 4 promotes chemoresistance of colorectal cancer by affecting lipid droplet synthesis and assembly. *World J Gastroenterol* 2023; 29(35): 5104-5124

**URL:** <https://www.wjgnet.com/1007-9327/full/v29/i35/5104.htm>

**DOI:** <https://dx.doi.org/10.3748/wjg.v29.i35.5104>

## INTRODUCTION

The regenerating gene 4 (*REG4*) gene family encodes secretory calcium-dependent lectins, which act as acute phase reactants, antiapoptotic factors, and growth agents, and contributes to cellular proliferation and differentiation, inflammation, diabetes, and cancer. To date, human *REG* genes include *REG I* ( $I\alpha$  and  $I\beta$ ), *Reg III* (III and *HIP/PAP*), and *REG4*[1-3]. Of these, *REG4* is transcriptionally activated by several transcription factors (*e.g.*, Sp1 transcription factor, GATA binding protein 6 (GATA6) and caudal type homeobox 2), and the *REG4* protein targets, epidermal growth factor receptor (EGF) (EGFR)/Akt/activator protein-1 and Akt/glycogen synthase kinase-3 $\beta$ / $\beta$ -catenin/T cell factor-4 signaling pathways as a mitogen[4-8]. In pancreatic cancer cells, *REG4* also enhanced invasion by upregulating matrix metalloproteinase expression and macrophage polarization through the EGFR-Akt-c-AMP response element binding protein pathway[9,10]. In gastric cancer cells, the forced expression of *REG4* induced EGFR phosphorylation and inhibited 5-fluorouracil (5-FU)-induced apoptosis[11]. In ovarian cancer cells, *REG4* overexpression as well as recombinant (rh) *REG4* exposure prevented apoptosis, and enhanced proliferation, migration, and invasion with the hyperexpression of Wnt5a, p70s6k, survivin and vascular endothelial growth factor, and reduced expression of Bax[12].

In colorectal tissues, *REG4* mRNA-positive cells are mostly enteroendocrine and goblet cells. Adenomatous and cancer cells positive for *REG4* mRNA exhibited enterocyte-like, mucus-secreting, or undifferentiated features[13]. At the protein level, *REG4* expression was observed in the middle and outer parts of crypts and superficial epithelium, especially goblets[14]. Oue *et al*[15] found that the preoperative serum *REG4* concentration was not elevated in patients with colorectal cancer (CRC) at stages 0-III, but was significantly elevated in those at stage IV. Additionally, *REG4* expression was significantly linked to a worse prognosis in patients with CRC as an independent predictor[15-17]. *REG4* promoted migration and invasion of CRC cells *via* its carbohydrate-recognition domain in both autocrine and paracrine manners, which was significantly decreased by anti-*REG4* antibody[18,19]. Nanakin *et al*[20] found that *REG4* expression was stimulated by tumor necrosis factor (TNF)  $\alpha$ , EGF, basic fibroblast growth factor, and hepatocyte growth factor in colon cancer cells, and then promoted cell proliferation and resistance to H<sub>2</sub>O<sub>2</sub>-induced apoptosis. As for therapy resistance, *REG4* was markedly related to chemoresistance. Violette *et al*[13] discovered that *REG4* protein was strongly expressed in

chemoresistant rectal cancer cells, but expressed weakly in drug-sensitive rectal cancer cells. *REG4* expression was found to correlate with  $\gamma$ -radiation sensitivity in rectal cancer patients receiving radiotherapy[21]. In radiochemotherapy-sensitive CRC cells, *REG4* expression was downregulated, while it was increased in radiochemoresistant cells[22].

Leukocytes have been found to play an important role in shaping the immune microenvironment of CRC[23-25]. Among them, the interleukin (IL)-22-mediated positive effect depended on its ability to induce neutrophil chemokines into the tumor microenvironment[24]. The proinflammatory cytokine group factors (*e.g.* TNF- $\alpha$ , IL-6, IL-12 and IL-23) promoted tumor cell survival, induced angiogenesis, and facilitated cell migration by exerting antiapoptotic activity[25]. Immune checkpoint molecules, including programmed death ligand 1/2, B7-1/2, B7-acetyl-histone 3 (H3), B7x, V-domain Ig suppressor of T cell activation and galectin-9, were found to be effective regulators of immune activation, which play a key role in immune escape of CRC[26]. Jang[27] found that T regulatory (Treg) cells gradually increased, while CD8<sup>+</sup> T cells and CD8<sup>+</sup> T cell/Treg cell ratio decreased during progression of CRC. Infiltration of T cells differed between tumor regions, and the proportion in the central region of CRC was the lowest. Unfortunately, there is no report on the relationship between *REG4* and the microenvironment in CRC.

In our previous study, *REG4* expression gradually decreased from gastric intestinal metaplasia, adenoma, and cancer to gastritis at either the mRNA or protein level[28]. Serum *REG4* levels were elevated in patients with gastric cancer, compared to those in healthy individuals. Our group subsequently developed a mouse anti-*REG4* monoclonal antibody for immunohistochemistry, and found that *REG4* was downregulated in CRC[3]. To understand the roles of *REG4* in tumorigenesis and subsequent progression of CRC, we undertook meta-analysis, and bioinformatics, pathological and serological analyses to explore the clinicopathological and prognostic significances of *REG4* in CRC. The effects of *REG4* on the phenotypes of CRC cells were also studied with the detailed mechanisms clarified. We also investigated the impacts of *REG4* on lipid droplet formation and chemoresistance of CRC cells and the regulatory effect of *REG4* on the key lipogenic enzymes in CRC cells.

## MATERIALS AND METHODS

### Cell culture and transfection

A CRC cell line (DLD-1) was generously donated by Prof. Toshiro Sugiyama, Department of Gastroenterology, University of Toyama, Japan. This line was maintained in RPMI 1640 growth medium supplemented with 11% fetal bovine serum (FBS), 90 U/mL penicillin, and 90  $\mu$ g/mL streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. The cells were transfected at 75% confluence 20-24 h after seeding on culture dishes (Qiagen, Hilden, Germany). The cells were transfected with pcDNA3.1-*REG4* [full-length (FL)-*REG4*], pcDNA3.1-*REG4* [nonsignal peptide (NSP)-*REG4*] or siRNA against histone deacetylase [si histone deacetylase (HDAC); Santa Cruz Biotechnology, Dallas, TX, United States] using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, United States). The cells were treated with high glucose (4.5 mg/L), recombinant human *REG4* protein (rh*REG4*; R and D Systems, Minneapolis, MN, United States) or anti-*REG4* antibody (R and D Systems). The cells were also exposed to 5-FU (thymidylate synthetase inhibitor); cisplatin (DDP), DNA crosslinker; suberoylanilide hydroxamic acid (SAHA); HDAC inhibitor; cycloheximide (CHX), selective inhibitor of protein synthesis to study the degradation and stability of proteins; MG132 (proteasome inhibitor to suppress proteasomal degradation of proteins); acetyl-CoA carboxylase 1 (ACC1) or ATP-citrate lyase (ACLY) inhibitor (Abmole, Chicago, United States). Centrifuged cells were washed with phosphate-buffered saline (PBS), and total protein or RNA was extracted. Some cells were subjected to proteasome extract using a Minute Nuclear or cytosolic proteasome enrichment kit (Invent Biotechnologies, Plymouth, MN, United States). For HDAC silencing, we tested at least two HDAC-specific siRNAs to control for off-target effects.

### Assessment of cell proliferation by tetrazolium salt (MTT) assay

As a measure of cell proliferation, MTT was reduced to formazan in a mitochondrial-dependent manner. We seeded cells in 96-well plates. After culture for 24 or 48 h in a 37 °C incubator, 50  $\mu$ L MTT reagent (5 mg/mL) was added to each well. After incubation for 4 h, 150  $\mu$ L dimethyl sulfoxide was added to each well for coloration. After vigorous shaking of the plates for 30-min to achieve complete solubilization, the optical density was measured on a microplate reader (Model 680; Bio-Rad, Hercules, CA, United States) at a wavelength of 490 nm. We normalized the OD 490 values to the viability of control cells that were not treated with reagents or siRNA. Normalized viability (%) = (OD490 of treated cells/OD490 of untreated control cells)  $\times$  100. The normalized viability values were plotted to show the growth inhibition effects of treatments.

### Apoptosis assay

We detected phosphatidylserine externalization (on the cell membrane) using fluorescein isothiocyanate (FITC)-labeled Annexin V (Immunotech, Marseille, France) and propidium iodide (PI) to identify early apoptosis. After treatment, any remaining intact cells were collected, washed with cooled PBS at 4 °C and centrifuged at 700  $\times$  g for 3 min. The cell suspension (490  $\mu$ L) was gently mixed with FITC-labeled Annexin-V (5  $\mu$ L) and PI (5  $\mu$ L). After incubation at 4 °C for 10 min in the dark, the cell suspension was detected by flow cytometry.

### Wound healing assay

Cells (5.5  $\times$  10<sup>5</sup> per well) were plated in six-well culture plates. When the cells reached 75% confluence, they were scraped with a tip, washed four times in PBS to reduce the number of broken cells, and cultured in FBS-free RPMI 1640 medium.

We measured the area of the scratch immediately after wounding (0 h) and 24 and 48 h later using Image J. The percentage wound closure was calculated as: (Area of scratch at 0 h-area of scratch at 24 and 48 h)/area of scratch at 0 h) × 100. The values were plotted to show differences in migration between conditions.

### Cell migration and invasion assays

Assays for migration were conducted by suspending  $1.3 \times 10^5$  cells per 200  $\mu$ L in FBS-free RPMI 1640 and seeding the upper chamber of each Transwell (BD Biosciences, Franklin Lakes, NJ, United States). As a chemoattractant, 11% FBS was added to each lower compartment. After incubation for 24 h, we scrubbed the upper surface of the Transwell membrane, washed the Transwell chamber with PBS three times and fixed cells in 100% cold methanol. The membrane was stained with crystal violet for several minutes. For the invasive assay, the same process as above was used except that each Transwell insert was coated with Matrigel (BD Biosciences).

### Nile red staining

Cells were cultured on coverslips for 12 h, fixed in 4% paraformaldehyde for 28 min and stained with Nile red (Invitrogen, Carlsbad, CA, United States; 1: 1000) for 13 min. Finally, we stained slides with 4',6-diamidino-2-phenylindole (DAPI) and covered them with SlowFade® Gold anti-fade reagent (Thermo Fisher Scientific). The software programs, Image J and Icy, were used to acquire and analyze images.

### Immunofluorescence

Cells were cultured on glass slides, fixed for 10 min with 4% formaldehyde, and permeabilized for 10 min with 0.5% Triton X-100. Cells were rinsed with PBS and treated with anti-goat REG4 (R and D Systems; 1: 100) antibody for 3 h. The cells were incubated with Alexa Fluor 488 (green) anti-goat immunoglobulin G (IgG) (Invitrogen) for 50 min. Slides were mounted using SlowFade® Gold anti-fade reagent after being stained with DAPI.

### Chromatin immunoprecipitation (ChIP)

A Magna ChIP™ G kit (Millipore, Burlington, MA, United States) was used to conduct ChIP. After IP of anti-acetyl (AC) H3, anti-AC-histone 4 (H4), anti-HDAC, anti-inhibitor of growth protein (ING) 5 or anti-sterol-regulatory element binding protein 1 (SREBP1) antibody (Supplementary Table 1), we used ACLY (5'-AATCGCGGGGCGGTTCTC-3', melting temperature (TM): 57.9 °C; 5'-CGACGAACCCCGCAAAATC-3', TM: 55.4 °C, -43 bp to + 81 bp) primers or ACC1 (5'-GCCCCGAATGGCAGATCC-3', TM: 55.7 °C; 5'-GCTCAGCGGCAGCCAATG-3', TM: 57.4 °C; -33 bp to + 54 bp) primers for polymerase chain reaction (PCR). IgG was used as a negative control and anti-polymerase II as a positive control. In 20  $\mu$ L of mixture, DNA was amplified and separated on a 2% agarose gel.

### Co-immunoprecipitation

Seven micrograms of primary antibody (Supplementary Table 1) was added to > 1 mg protein and subjected to rotation at 4 °C overnight. One hundred microliters of agarose A beads were added, and the mixture was rotated at 4 °C overnight. To exclude nonspecific binding proteins, the beads were centrifuged and washed with 1% NP40 Lysis buffer four times. The pellet was mixed using 50  $\mu$ L 2 × SDS sample buffer, and heated at 100 °C for 10 min. The sample supernatant was used for western blotting.

### Patients

Colorectal primary cancers ( $n = 796$ ), adenomas ( $n = 62$ ), non-neoplastic mucosa [(NNM),  $n = 667$ ] and metastatic cancers in lymph nodes ( $n = 179$ ) were sampled at the First Affiliated Hospital of Jinzhou Medical University between 2013 and 2022. One hundred cases of CRC and adjacent NNM were obtained from Liaoning Cancer Hospital and stored at -80 °C until RNA and protein extraction. Eighty patients with CRC and 50 healthy volunteers were enrolled to determinate the serum REG4 level at Liaoning Cancer Hospital and The Affiliated Hospital of Chengde Medical University. Before surgery, none of the patients had undergone radiotherapy or chemotherapy. The ethics committee of our institution authorized the research plan after giving unanimous approval to utilize tumor tissues and patient serum for clinical research.

### Pathology and tissue microarray

All tissues were preserved in 10% neutral formalin, embedded in paraffin, and sectioned into 4-mm pieces. Hematoxylin and eosin (HE) staining was used to validate the histological diagnosis and other microscopic features of these sections. In HE-stained sections, representative portions of solid tumors were selected under a microscope. Using a tissue microarray, a 2-mm tissue core from each donor block was punched out and transferred to a recipient block with a maximum of 48 cores (Azumaya kin-1, Nagoya, Japan). From the recipient block, 4- $\mu$ m-thick sections were sequentially cut and placed on glass slides coated with poly-lysine.

### Western blotting

One hundred and seventy microgram samples of denatured protein were separated on 12% SDS-PAGE and then transferred to a Hybond membrane (Amersham, Chicago, IL, United States). The membrane was blocked overnight in 4.5% skimmed milk in Tris-buffered saline with Tween 20 (TBST). The membrane was treated with primary antibody for immunoblotting for 1 h (Supplementary Table 1). Following a TBST rinse, it was incubated for 1 h with anti-goat, anti-mouse or anti-rabbit IgG conjugated to [horseradish peroxidase(HRP); Dako, Glostrup, Denmark]. Bands were observed



using C300 imaging system (AZURE, Peking, China) and ECL-Plus detection reagents (Santa Cruz Biotechnology).

### Quantitative reverse transcription polymerase chain reaction (RT-PCR)

CRC cells or tissues were used to extract total RNA with a Qiagen RNase Mini Kit. avian myeloblastosis virus transcriptase and a random primer were used to create cDNA from 2 mg total RNA (Takara, Kusatsu, Japan). The primers for *REG4* were forward: 5'-CCTTTCCACAGTATCCTTCTCCCT-3', TM: 58.4 °C and reverse: 5'-TATGGC-CAAAGACCCAGCTGTT-3', TM: 58.2 °C (104 bp). The primers for *ACLY* were forward: 5'-AAACTGTGG-GTCTTTACTCG-3', TM: 53.8 °C and reverse: 5'-GGATGACGATACAGCCCCTG-3', TM: 55.6 °C (147 bp). The primers for *ACC1* were forward: 5'-GCTGGTCCACATGAACAGG-3', TM: 53.8 °C and reverse: 5'-GCCTTCTGGATATTCAGGAC-TTT-3', TM: 54.5 °C (91 bp). The primers for *GAPDH* were forward: 5'-CAATGACCCCTTCATTGACC-3', TM: 52.0 °C and reverse: 5'-TGGAAGATGGTGATGGGATT-3', TM: 51.7 °C (135 bp). RT-PCR amplification was performed using a SYBR Premix Ex Taq™ II kit (Takara).

### Immunohistochemistry

After deparaffinization with xylene and dehydration with alcohol, successive slices in a target retrieval solution were microwaved for 17 min (Dako). H<sub>2</sub>O<sub>2</sub> at 3% in methanol was used to block endogenous peroxidase activity. To stop nonspecific binding, 4% bovine serum albumin was administered for 5 min. The sections were treated with anti-goat conjugated to horseradish peroxidase (Dako, 1: 100) antibodies for 18 min after being incubated with goat anti-human *REG4* antibody (R and D Systems, 1: 50) for 17 min. To enable previously reported intermittent irradiation, all incubations were carried out in a microwave oven[28]. *REG4* immunostaining was localized in the cytoplasm. For immunohistochemistry, according to the degree of color development of cell positive markers, it was divided into: Blue, negative; pale yellow, weakly positive; brown, moderately positive; dark brown, strongly positive. From five typical fields in each region, 100 cells were randomly chosen and counted by two independent observers blinded to the samples (Zhang CY and Zheng HC). Semiquantitative two-tier grading was used to determine the positive proportion of counted cells: Positive, 6%-100% and negative, 0%-5%.

### ELISA

We performed an ELISA (Gelatin, Shanghai, China) to determine serum *REG4* concentration. One hundred microliters of standard or serum sample was incubated at 4 °C overnight on polystyrene microtiter plates coated with anti-*REG4* antibody. Following the removal of the liquid, we added 100 µL biotin-antibody working solution to each well, which was then incubated at 37 °C for 2 h. After aspirating the liquid, 100 µL HRP-avidin working solution was added to each well, followed by washing three times with 350 µL wash buffer, and incubation at 37 °C for 1 h. The plates were washed again three times with wash buffer, followed by incubation with 90 µL tetramethylbenzidine substrate at 37 °C for 30 min. We dispensed 50 µL stopping solution to each well and measured the absorbance at 405 nm. Recombinant *REG4* (0.312-20 ng/mL) was used as the reference standard.

### Meta-analysis

PubMed, Web of Science, BIOSIS, and SciFinder were used to search the literature up to March 14, 2022. The following search phrases were entered: (Colorectal or rectal or colon or rectum) and (*REG4* or *REG IV*) and (cancer or carcinoma or adenocarcinoma). No limitations on language or publication year were placed on the search. Inclusion criteria for studies were: (1) Studies that used immunohistochemistry to detect changes in *REG4* expression in CRC; and (2) studies that used immunohistochemistry to relate *REG4* expression to pathobiological behavior and CRC prognosis. Exclusion criteria included: (1) Abstracts, comments, reviews and meeting reports; (2) duplication of previous publications; (3) western blotting, RT-PCR, cDNA microarray, or transcriptomic sequencing for *REG4* expression; and (4) lack of sufficient information. Two reviewers (Zhang CY and Zheng HC) independently gathered data from all relevant articles and evaluated the quality of the included studies using the Newcastle-Ottawa Scale (<http://www.ohri.ca/programs/clinicalepidemiology/oxford.htm>). For survival analysis, we extracted data from Kaplan-Meier curves using an Engauge Digitizer program. We calculated the hazard ratios (HRs) and accompanying 95% confidence intervals (CIs). Twelve papers regarding the correlation between *REG4* expression and cancer risk, and clinicopathological or prognostic factors of CRC were found in PubMed, Web of Science, BIOSIS Citation Index, SciFinder, and CNKI (Supplementary Table 2). Samples of normal colorectal mucosa were only included in four papers[3,29-31]. In 12 investigations, a comparison was made between *REG4* expression and the clinicopathological features of CRC[3,15,17,29-37]. Finally, we covered the importance of *REG4* expression for prognosis in five papers [15,17,30,35,36].

### Bioinformatics analysis

We used Oncomine ([www.oncomine.org](http://www.oncomine.org), keywords: *REG4*, Skrzypczak, Sabates-Bellver) to analyze the *REG4* expression level. The Cancer Genome Atlas (TCGA)-assembler in R program retrieved the *REG4* expression and clinicopathological data for a total of 362 patients with CRC from the TCGA database (keywords: *REG4*). Using the Xiantao platform (<https://www.xiantaozi.com/>, keywords: *REG4*) and The University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN) database (<http://ualcan.path.uab.edu>, keywords: *REG4*), we analyzed the expression, methylation, relevant genes, and signaling pathways of the *REG4* gene. A Kaplan-Meier plotter (<http://kmplot.com/>, keywords: 223447\_at) was used to evaluate the prognostic value of *REG4*. Additionally, we discovered the genes that were differentially expressed by Xiantao and subjected these to protein-protein interaction (PPI) network analysis and a search of critical hub genes. Subsequently, gene ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA) analyses were conducted on these genes.

## Statistics analysis

Hardy-Weinberg equilibrium was assessed in each study's control group using the  $\chi^2$  test. A Z test was used to establish the statistical significance of the pooled odds ratios. A fixed effect model was applied if there was no discernible heterogeneity. Instead, a random effect model was used to analyze prognostic analysis. An  $I^2$  test was used to quantify the heterogeneity impact. To measure the asymmetry of the funnel plot and evaluate publication bias, Begg's and Egger's tests were used to quantify this. Meta-analyses were carried out using Revman software 5.3, and Student  $t$  tests were used with SPSS 10.0 to handle data from the TCGA database. A log-rank statistic was used to compare survival curves and create Kaplan-Meier survival charts. Cox's hazard proportional analysis was used for multivariate survival analysis. Statistical significance was defined as two-sided  $P < 0.05$ .

## RESULTS

### Clinicopathological and prognostic significance of REG4 mRNA expression in CRC

The expression of REG4 mRNA was higher in colorectal adenoma or CRC than in normal tissues ( $P < 0.05$ ) using quantitative RT-PCR (Supplementary Figure 1A), Xiantao (Supplementary Figure 1B) and Oncomine (Supplementary Figure 1C) datasets. In TCGA data, REG4 mRNA expression was higher in colonic than rectal cancer ( $P < 0.05$ ) (Supplementary Figure 1D). A positive correlation was noted with microsatellite instability status and BRAF mutation, but a negative correlation was found with lymph node metastasis, distant metastasis, and tumor-node-metastasis (TNM) staging in CRC ( $P < 0.05$ ) (Supplementary Figure 1D). Mucinous adenocarcinoma showed higher REG4 mRNA expression than the other adenocarcinomas ( $P < 0.05$ ) (Supplementary Figure 1E). Kaplan-Meier analysis demonstrated a significantly positive association between REG4 mRNA expression and the overall survival rate of patients with cancer ( $P < 0.05$ ) (Supplementary Figure 1F), even though Cox's proportional hazards analysis indicated that this relationship was not independent ( $P > 0.05$ ) (643 patients, HR univariate/multivariate analysis 1.436, 95% CI 0.911-2.039). On the basis of a Kaplan-Meier plot, REG4 mRNA expression had a positive correlation with overall survival of female or white patients, and those with a high mutation burden ( $P < 0.05$ ) (Supplementary Figure 1G). REG4 methylation [transcription start site (TSS)-289, TSS-46, TSS + 45 and TSS + 2831] and mRNA expression were negatively correlated in CRC ( $P < 0.05$ ) (Supplementary Figure 2A). The REG4 methylation level was lower in colon cancer than in normal mucosa ( $P < 0.05$ ) (Supplementary Figure 2B), in stage 2 than in stage 4 cancer, in mucinous adenocarcinoma than adenocarcinoma, and in tumor protein p53 nonmutant than mutant cancer ( $P < 0.05$ ) (Supplementary Figure 2C).

### REG4-related genes and signaling pathways in CRC

We identified distinct genes in the low and high expression groups of REG4 mRNA in CRC using a xiantao platform, and constructed a volcanic map (Supplementary Figure 3A). KEGG analysis showed that the top signaling pathway mainly included chemokine activity, taste receptor, protein-DNA and a DNA-packing complex, and nucleosome and chromatin ( $P < 0.05$ ) (Supplementary Figure 3B). GSEA showed that the top signaling pathways were principally composed of a generation of second messenger molecules: programmed cell death protein 1 signaling, HDAC and histone acetyl transferase, and epigenetic regulation and DNA methylation ( $P < 0.05$ ) (Supplementary Figure 3C). The upregulated genes were CCL19, CCL25, CIDEA, CMA1 and PLA2G2D, and downregulated genes were ANGPTL3, CRP, H2BC10, H4C3 and H4C6 ( $P < 0.05$ ) (Supplementary Figure 3D). Cytoscape software was utilized to determine the top 10 nodes by degree and STRING software to identify the PPI pairings (Supplementary Figures 4A and B). According to a Xiantao database, CD3D, 3D3G, CD4, HLA-DRA, ZAP70, ITK, CD3E, CD247, CD28 and HLA-DRB1 were less expressed in CRC than normal tissues ( $P < 0.05$ ) (Supplementary Figure 4C).

According to the Xiantao database, genes positively correlated with REG4 in CRC are shown in Supplementary Figure 5A ( $P < 0.05$ ). These genes were mainly involved in transcription repression and activation by DNA binding, and lung epithelial differentiation ( $P < 0.05$ ) (Supplementary Figure 5B). Genes negatively correlated with REG4 in CRC are shown in Supplementary Figure 5C ( $P < 0.05$ ), and were principally involved in the insulin signaling pathway, sugar metabolism and transfer, and neurotransmitter receptors ( $P < 0.05$ ) (Supplementary Figure 5D). Of the positively correlated genes (Supplementary Figure 5E), AGR2, B3GNT6, CREB3L1, CTSE, FAM177B, FCGBP, FFAR4, MUC2 and SPDEF were less expressed in CRC than normal mucosa ( $P < 0.05$ ), while GRB2 was more expressed in CRC than normal mucosa ( $P < 0.05$ ). Of the negatively correlated genes (Supplementary Figure 5F), LY6G6E, LY6G6D, FAM27B, PFDN4, PPP1R3D, LY6G6F, RNF43, POFUT1 and DDX27 were more expressed in CRC than normal mucosa ( $P < 0.05$ ), but MT-RNR1 was less expressed in CRC than normal mucosa ( $P < 0.05$ ).

### Relationship between REG4 mRNA expression and immune cell infiltration in CRC

According to xiantao, the infiltration of mast cells, T cells, CD8 T cells, cytotoxic T cells, T helper (Th)1, Th2 and Th17 cells, T follicular helper (TFH) cells, TReg cells, natural killer (NK) CD56<sup>bright</sup> cells, B cells, interstitial dendritic cells (iDCs), and activated DCs (aDCs) in CRC were positively correlated with REG4 mRNA expression ( $P < 0.05$ ) (Supplementary Figure 6). Meanwhile, T central memory (Tcm) cell infiltration was negatively correlated with REG4 mRNA expression ( $P < 0.05$ ) (Supplementary Figure 6).

### Clinicopathological and prognostic significance of REG4 protein expression in CRC

Compared to normal mucosa, REG4 protein expression was low in CRC ( $P = 0.03$ ) (Supplementary Table 3) and positively correlated with lymph node metastasis, TNM staging, and dedifferentiation of CRC ( $P < 0.05$ ) (Supplementary Tables 4-



6). The combined data from five datasets showed a strong correlation between REG4 expression and overall survival in patients with CRC (Supplementary Table 7) (HR = 0.54, 95%CI: 0.41-0.72,  $P < 0.0001$ ).

According to UALCAN (Supplementary Figure 7A), REG4 protein expression was higher in mucinous than nonmucinous adenocarcinoma ( $P < 0.05$ ), and negatively correlated with chromatin modifier, p53/Rb, and the HIPPO signaling pathway ( $P < 0.05$ ). Expression of REG4 protein was higher in CRC than in NNM, according to western blotting ( $P < 0.05$ ) (Supplementary Figure 7B). Serum REG4 level was higher in patients with CRC than in healthy volunteers after modification by body surface area ( $P < 0.05$ ) (Supplementary Figure 7C). Immunohistochemically, REG4 expression was higher in colorectal NNM than adenoma and primary cancer, and in primary than metastatic cancer ( $P < 0.001$ ) (Supplementary Figure 7D and E). Mucinous adenocarcinoma showed higher REG4 expression than well-, moderately and poorly differentiated adenocarcinomas ( $P < 0.05$ ) (Supplementary Table 8).

### Effects of REG4 on the phenotype of CRC cells

According to qPCR ( $P < 0.05$ ) (Supplementary Figure 8A), western blotting (Supplementary Figure 8B), immunofluorescence (Supplementary Figure 8C), and ELISA ( $P < 0.05$ ) (Supplementary Figure 8D), DLD-1 cells were successfully transfected with FL-REG4 and NSP-REG4 plasmids, as shown by higher REG4 mRNA and protein expression in transfectants than parental cells. After treatment with rhREG4, the viability of DLD-1 cells was increased in a dose-dependent manner ( $P < 0.05$ ) (Figure 1A). Exposure to anti-REG4 antibody decreased the cell viability of FL-REG4-overexpressing DLD-1 cells in a dose-dependent manner ( $P < 0.05$ ) (Figure 1B). Treatment with rhREG4 or FL-REG4 overexpression resulted in high viability ( $P < 0.05$ ) (Figure 1C), antiapoptosis ( $P < 0.05$ ) (Figure 1D), migration and invasion ( $P < 0.05$ ) (Figures 1E and F) in comparison with untreated DLD-1 cells ( $P < 0.05$ ). Additionally, FL-REG4 transfection and rhREG4 treatment enhanced the expression of REG4, EGFR-Tyr992, -Tyr1068, -Tyr1148, -Tyr1173, phosphorylated phosphoinositide 3-kinase (p-PI3K), p-Akt, nuclear factor (NF)- $\kappa$ B, p-NF- $\kappa$ B, Bcl-2, and Bcl-X/L compared with untreated DLD-1 cells by western blotting ( $P < 0.05$ ) (Figure 1G). However, anti-REG4 antibody blocked the effects of FL-REG4 overexpression (Figure 1B-G). The effects of NSP-REG4 on aggressive phenotypes and their related protein expression in DLD-1 cells were not detectable, which differed from the effects of FL-REG4 expression and were similar to those of REG4-nontransfected CRC cells (Figure 1C-F and H).

### Effects of REG4 on chemoresistance and droplet formation of CRC cells

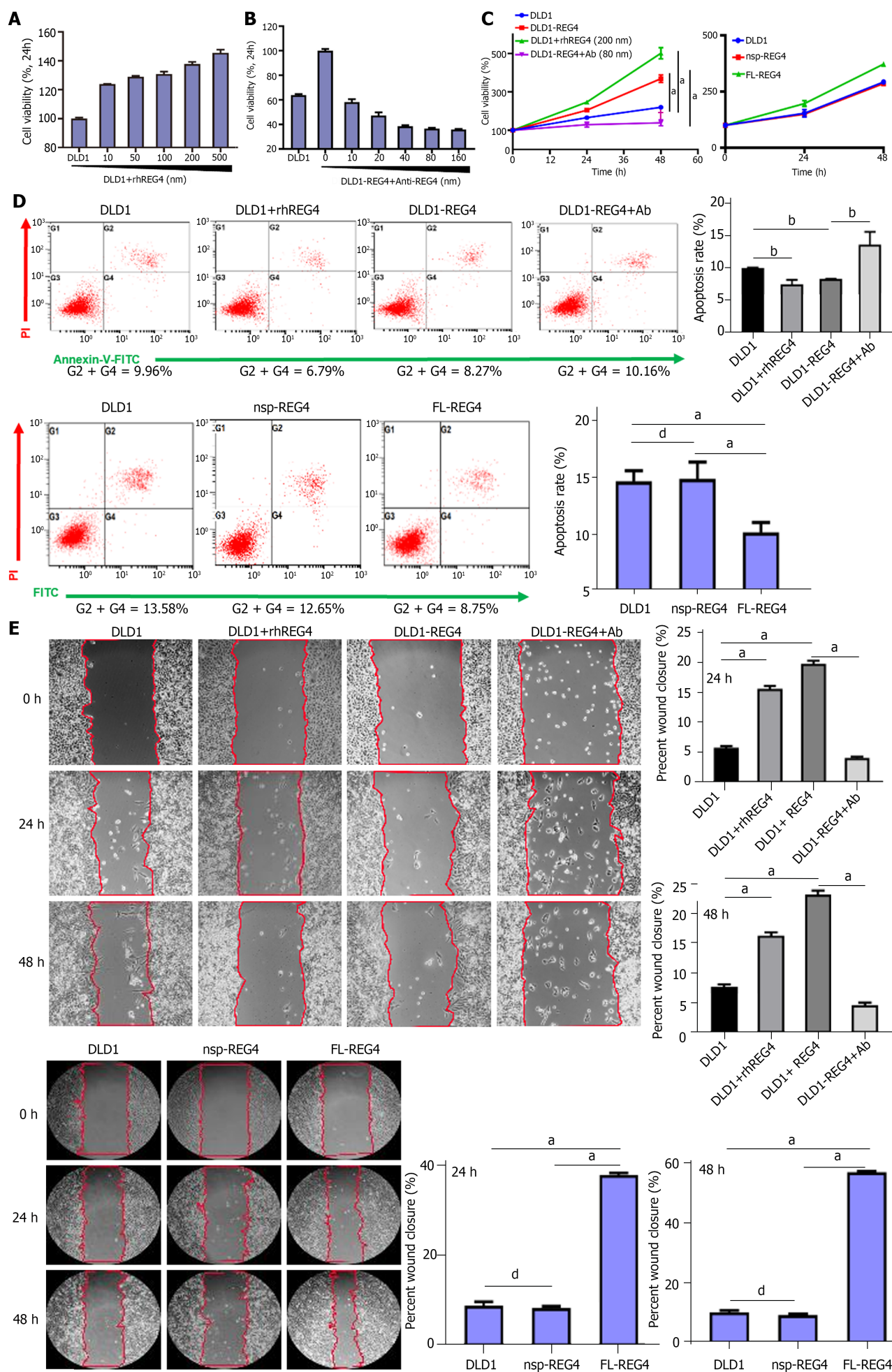
RhREG4 treatment and FL-REG4 overexpression caused DLD-1 cells to become resistant to DDP and 5-FU ( $P < 0.05$ ) (Figure 2A), while anti-REG4 antibody reversed the chemoresistance of REG4-overexpressing DLD-1 cells ( $P < 0.05$ ) (Figure 2A). rhREG4 treatment and FL-REG4 increased the formation of intracellular lipid droplets, as shown by Nile red staining ( $P < 0.05$ ) (Figure 2B). REG4 overexpression was observed in chemoresistant DLD-1 cells. FL-REG4 overexpression enhanced the expression of acyl coenzyme A-cholesterol acyltransferase (ACAT), perilipin 5, and tail-interacting protein (TIP) 47, but weakened expression of ACLY, ACC1, p-ACC1, HDAC, adipose differentiation-related protein (ADRP), cell-death-inducing DFF45-like effector (CIDE) A, B and C, AC-H3, AC-H4, ING5, and SREBP1 in DLD-1 cells (Figure 2C). High-glucose treatment significantly increased lipid droplet formation in DLD-1 cells, which was markedly suppressed by ACLY or ACC1 inhibitor ( $P < 0.05$ ) (Figure 2D). DLD-1 cells treated with rhREG4, FL-REG4 transfection, or high glucose had decreased chemosensitivity to 5-FU and DDP, but DLD-1 cells treated with REG4 antibody, ACC1 inhibitor or ACLY inhibitor had increased chemosensitivity to 5-FU and DDP (Figure 2E). ACC1 or ACLY inhibitor reversed the insensitivity of cells to 5-FU and DDP in the high-glucose treatment group ( $P < 0.05$ ).

### REG4 weakened transcription of ACLY and ACC1 via disassociating AC-H3-AC-H4-HDAC-ING5-SREBP1 complex

In DLD-1 cells, FL-REG4 overexpression weakened the interaction of AC-H3, AC-H4, HDAC, SREBP1 and ING5 proteins with the ACLY or ACC1 promoter, according to ChIP ( $P < 0.05$ ) (Figure 3A). FL-REG4 overexpression also weakened the interaction of the five proteins ( $P < 0.05$ ) (Figure 3B). Overexpression of FL-REG4 decreased ACLY and ACC1 mRNA expression ( $P < 0.05$ ) (Figure 3C), and increased lipid droplet formation ( $P < 0.05$ ) (Figure 3D), and increased the half maximal inhibitory concentration (IC<sub>50</sub>) of cells for 5-FU and DDP ( $P < 0.05$ ) (Figure 3E). After SAHA (siHDAC inhibitor) treatment (2  $\mu$ M, 24 h) and siHDAC transfection, the interaction between AC-H3, AC-H4, SREBP 1, ING5, HDAC and ACC1, ACLY promoters was weakened compared with the control group ( $P < 0.05$ ) (Figure 3A), and the mRNA level of ACC1 and ACLY decreased ( $P < 0.05$ ) (Figure 3B). After SAHA treatment and siHDAC transfection, the fluorescence intensity of Nile red staining decreased compared with that in the control group ( $P < 0.05$ ) (Figure 3D), and the chemical sensitivity to 5-FU and DDP increased compared with that in the control group ( $P < 0.05$ ) (Figure 3E).

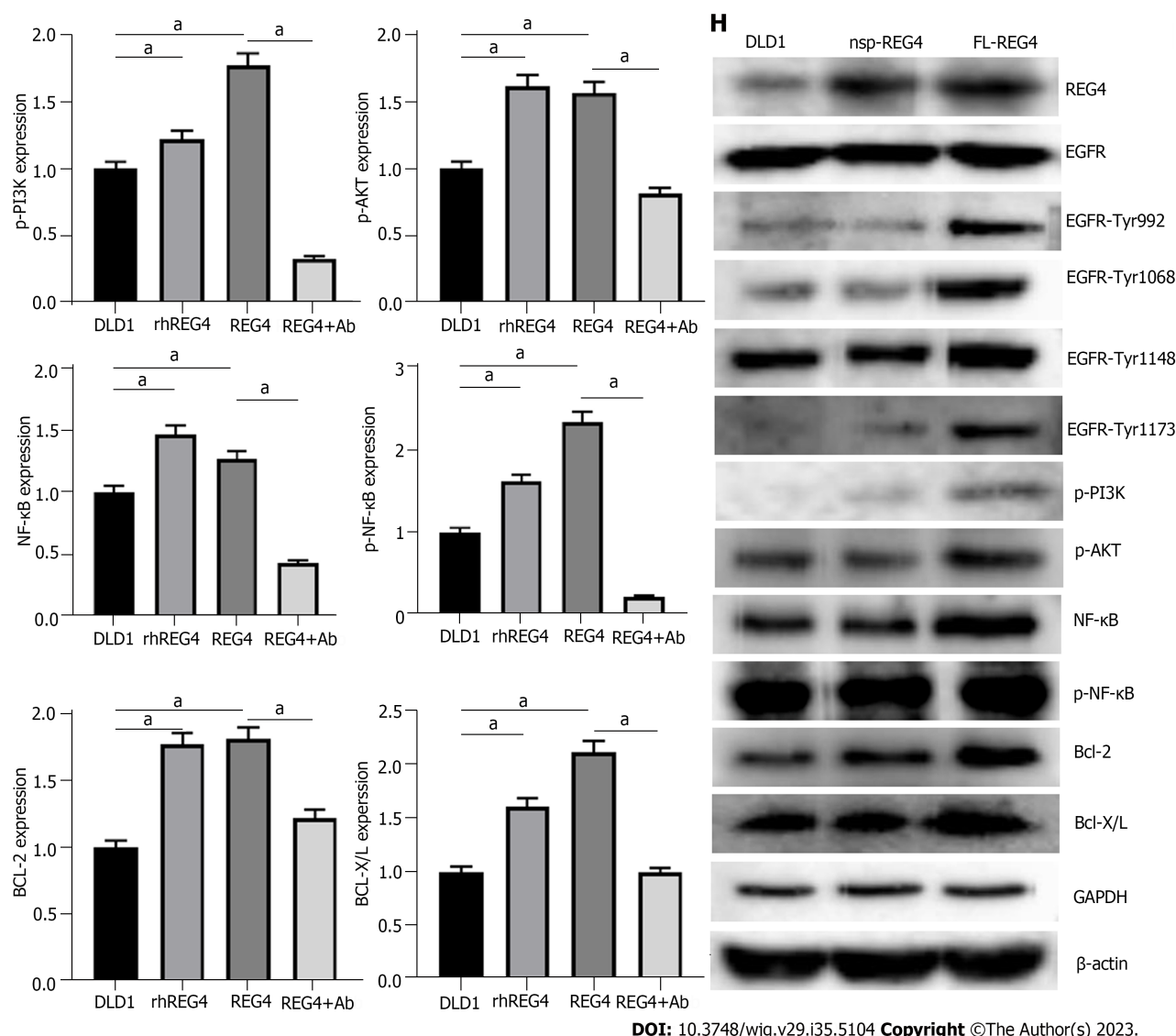
### REG4 destabilized ACLY and ACC1 proteins via proteasomal degradation

After treatment with CHX (used to inhibit the synthesis of new proteins), ACC1 and ACLY protein expression was lower in FL-REG4 transfectants than in DLD-1 cells ( $P < 0.05$ ) (Figure 4A). MG132 (proteasome inhibitor) increased protein expression of ACC1 and ACLY in DLD-1 cells and FL-REG4 transfectants, which was higher in DLD-1 cells than in FL-REG4 transfectants ( $P < 0.05$ ) (Figure 4B). Higher levels of ACC1 and ACLY proteins were noted in the nuclear proteasome of FL-REG4 transfectants than in DLD-1 cells, but this translocation phenomenon was not enhanced by MG132 (Figure 4C). Similar levels of ACC1 and ACLY expression were noted in the cytosolic proteasome of transfectants and parental cells, even after treatment with MG132 (Figure 4C). Regarding ubiquitin ligases, expression of COP1 E3 ubiquitin ligase (COP1), Cbl proto-oncogene (CBL) and NEDD4 like E3 ubiquitin protein ligase (NEDD4L) was higher in FL-REG4 transfectants than DLD-1 cells, but synoviolin 1 (SYVN1) and NEDD4 were lower in FL-REG4 transfectants than DLD-1 cells (Figure 4D). Co-immunoprecipitation showed that expression of ubiquitylated ACC1 and ACLY was higher in FL-REG4 transfectants than in DLD-1 cells, and pretreatment with a small dose of proteasome inhibitor MG132 (5  $\mu$ M,









**Figure 1** Effects of full-length regenerating gene 4 and nonsignal peptide regenerating gene 4 on phenotypes of DLD-1 cells. A: DLD-1 cells were incubated with increasing doses of recombinant human regenerating gene 4 (REG4) (rhREG4) (0-500 nmol/L) for 48 h and subjected to cell proliferation assay by tetrazolium salt (MTT) assay; B: Treatment with anti-REG4 antibody produced a dose-dependent decrease in cell number of full-length (FL)-REG4-overexpressing DLD-1 cells; C: MTT assay was used to detect the proliferation of DLD-1 cells transfected with FL-REG4 or nonsignal peptide (NSP)-REG4, and treated with rhREG4 or REG4 antibody; D: Apoptosis of DLD-1 cells transfected with FL-REG4 or NSP-REG4, and treated with rhREG4 or REG4 antibody detected by flow cytometry; E: Wound healing assay was used to detect migration of DLD-1 cells transfected with FL-REG4 or NSP-REG4, and treated with rhREG4 or REG4 antibody; F: Transwell assay was used to detect migration and invasion of DLD-1 cells transfected with FL-REG4 or NSP-REG4, and treated with rhREG4 or REG4 antibody; G: Compared with DLD-1 cells, western blotting showed that transfection with FL-REG4 and treatment with rhREG4 increased expression of epidermal growth factor receptor (EGFR)-Tyr992, Tyr1068, Tyr1148, Tyr1173, Akt, p-Akt, phosphorylated phosphoinositide 3-kinase (p-PI3K), nuclear factor (NF)-κB, p-NF-κB, Bcl-2 and Bcl-X/L. REG4 antibody inhibited the effect of FL-REG4 transfection; H: Western blotting showed that compared with parental cells, DLD-1 cells transfected with NSP-REG4 had no change in expression of EGFR-Tyr992, Tyr1068, Tyr1148, Tyr1173, AKT, p-AKT, p-PI3K, NF-κB, p-NF-κB, BCL-2, or BCL-XL. <sup>a</sup> $P < 0.001$ ; <sup>b</sup> $P < 0.01$ ; <sup>c</sup>No significance. Ab: Anti-REG4 antibody; REG4: Regenerating gene 4; rhREG4: Recombinant human regenerating gene 4; FL: Full-length; NSP: Nonsignal peptide; EGFR: Epidermal growth factor receptor; p-PI3K: Phosphorylated phosphoinositide 3-kinase; NF: Nuclear factor.

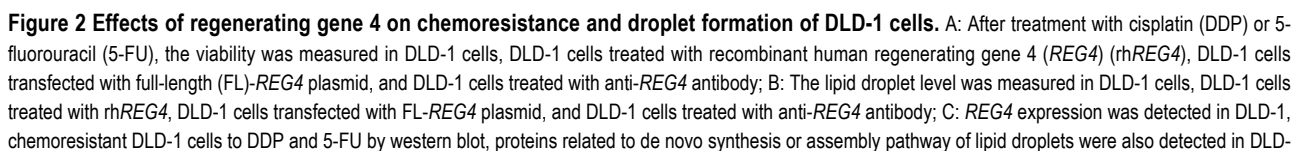
9 h) reduced ubiquitination of ACC1 and ACLY (Figure 4E). ACLY bound more to SYVN1, NEDD4 and NEDD4L in FL-REG4 transfectants than in DLD-1 cells, but bound less to CBL. ACC1 interacted more with COP1 in REG4 transfectants than in DLD-1 cells, but interacted less with CBL and SYVN1 (Figure 4E). MG132 pretreatment (5  $\mu$ M, 24 h) aggravated lipid droplet formation ( $P < 0.05$ ) (Figure 4F) and chemoresistance against 5-FU and DDP ( $P < 0.05$ ) (Figure 4G) in DLD-1 cells and FL-REG4 transfectants.

## DISCUSSION

The silencing of REG4 reduced cellular proliferation, but rhREG4 had the opposite effect since REG4 downregulated p21 (Cip1/WAF1) expression and upregulated cyclin D1 expression, which might promote G<sub>1</sub>/S transition[38,39]. Anti-REG4







1 cells and DLD-1 cells transfected with FL-*REG4*; D: Nile red staining was used to detect the level of lipid droplets in DLD-1 cells and DLD-1 cells transfected with FL-*REG4* after treatment with high glucose (HG), acetyl-CoA carboxylase 1 (ACC1) inhibitor and ATP-citrate lyase (ACLY) inhibitor; E: Tetrazolium salt assay was used to detect half maximal inhibitory concentration of DLD-1 cells for 5-FU and DDP when treated with HG, ACC1 and ACLY inhibitors, alone or in combination with the above conditions. <sup>a</sup>*P* < 0.001; <sup>b</sup>*P* < 0.01. Ab: Anti-*REG4* antibody; *REG4*: Regenerating gene 4; rh*REG4*: Recombinant human regenerating gene 4; ACC1: Acetyl-CoA carboxylase 1; ACCY: ATP-citrate lyase; AC-H3: Acetyl-acetyl-histone 3; H4: Histone 4; ACC1: Acetyl-CoA carboxylase 1; HDAC: Si histone deacetylase; ING5: Inhibitor of growth protein 5; SREBP1: Sterol-regulatory element binding protein 1; ACAT: A-cholesterol acyltransferase; ADRP: Adipocyte differentiation-related protein; CIDE: Cell-death-inducing DFF45-like effector; TIP: Tail-interacting protein; DDP: Cisplatin; 5-FU: 5-fluorouracil; HG: High glucose.

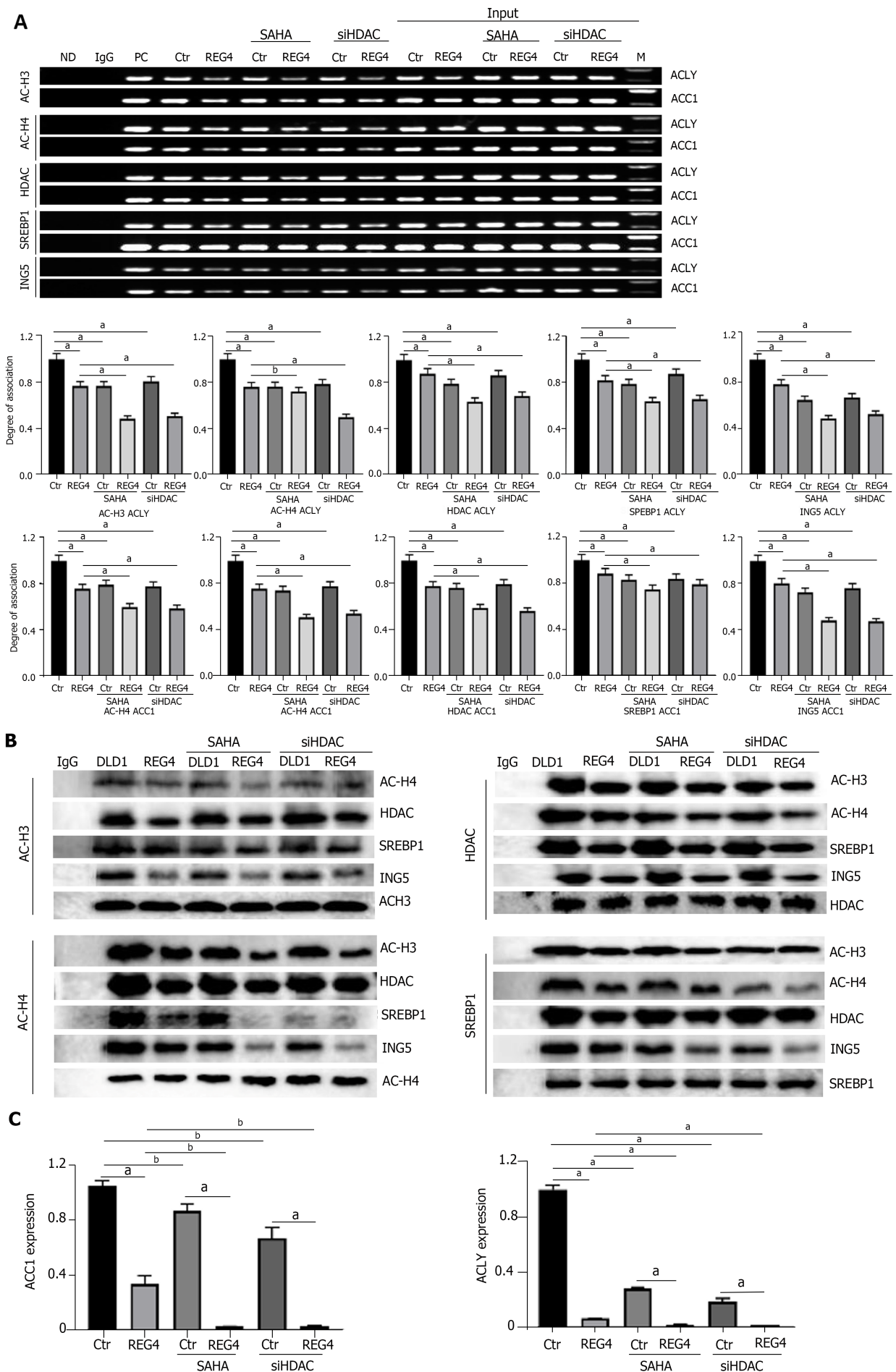
antibody dramatically reduced the autocrine and paracrine effects of secretory *REG4* on the ability of colon cancer cells to invade, migrate and proliferate[19]. *REG4* overexpression may cause resistance to the irradiation-induced apoptosis of colon cancer cells. Animal studies have shown that rh*REG4* increased the expression of the antiapoptotic genes, Bcl-2 and Bcl-xL, and survivin to shield normal intestinal crypt cells against irradiation-induced apoptosis[40].

We conducted bioinformatics analysis, meta-analysis, and pathological and serological studies to evaluate the clinicopathological and prognostic significance of *REG4* expression in CRC. In bioinformatics analysis, we used data of different sizes and from different sources to ensure data heterogeneity, and we used qRT-PCR to verify the reliability of bioinformatics analysis. We discovered that *REG4* mRNA expression was upregulated in CRC, but *REG4* protein expression was downregulated, suggesting that aberrant *REG4* expression could be used as a potential biomarker for colorectal tumorigenesis. In addition, we discovered a negative correlation between *REG4* promoter methylation and mRNA expression in CRC. The correlation of *REG4* methylation with mucinous subtypes or clinicopathological staging was opposite to that of *REG4* mRNA expression. Upregulated *REG4* mRNA expression might be due to promoter hypomethylation in CRC. High *REG4* mRNA expression was discovered in colorectal, pancreatic, hepatic, and prostate malignancies as well as in inflammatory epithelium, dysplasia, and malignant lesions of ulcerative colon tissues[20,41-45], suggesting that upregulated *REG4* mRNA was involved in the early malignant transformation of epithelial cells. Although upregulated *REG4* protein expression occurred in gastric cancer, ovarian cancer, glioma, pancreatic cancer, gallbladder carcinoma, and prostate cancer[12,28,46-49], lower *REG4* expression was found in CRC. In subsequent research, we will use more databases and updated data sets to analyze the expression of *REG4* in CRC to verify the consistency between our studies and strengthen our conclusions.

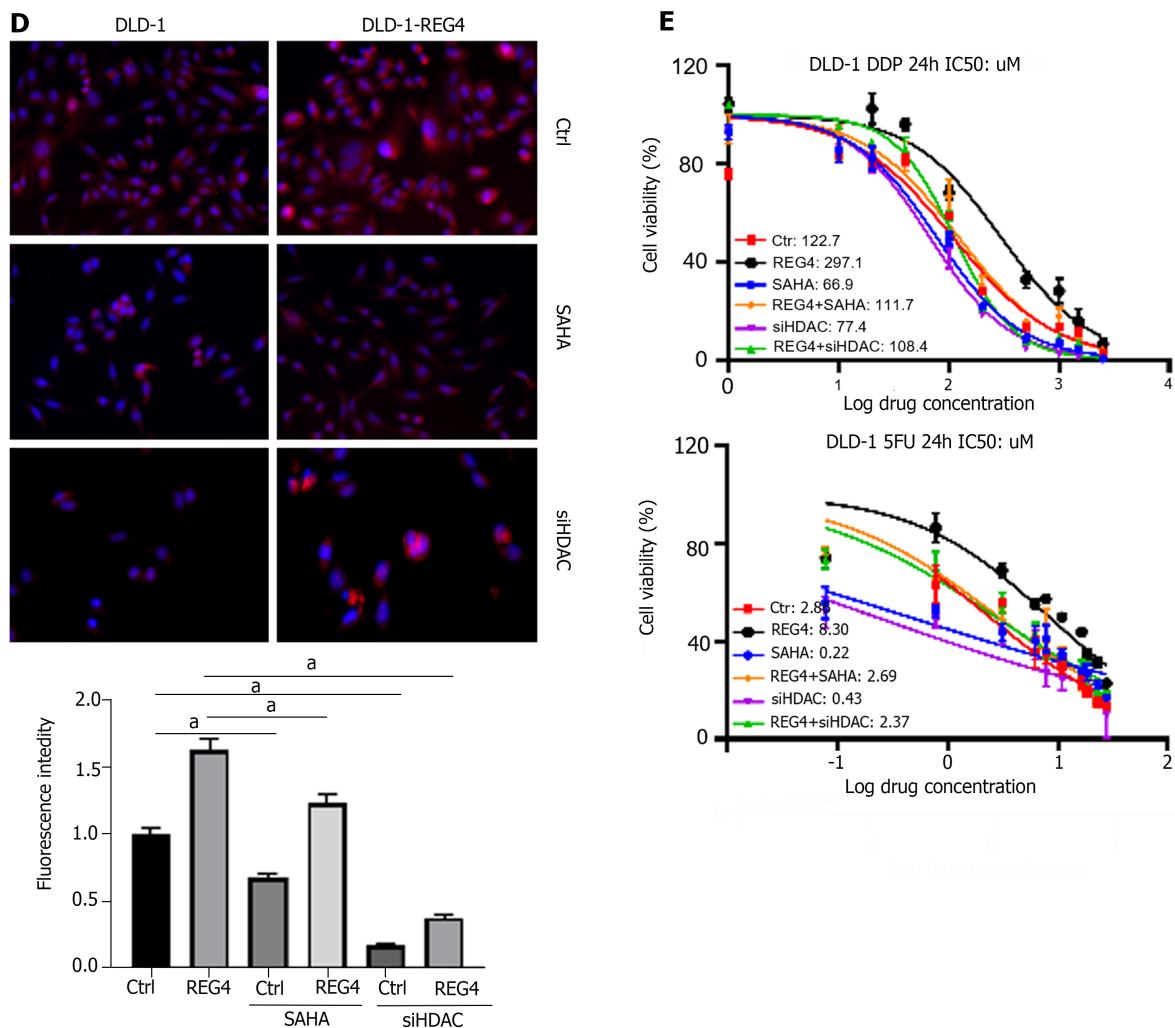
The tumor microenvironment of CRC is regulated by many factors, such as *CTLA-4*, and the altered expression of these factors can lead to changes in immune responses[50]. So far, there has been no systematic study on the relationship between *REG4* and immune microenvironment in CRC. Here, we studied the correlation between the expression of *REG4* mRNA and the infiltration of immune cells in CRC, and found that high expression of *REG4* was positively correlated with infiltration of mast cells, T cells, CD8 T cells, cytotoxic T cells, Th1, Th2 and Th17 cells, TFH cells, TReg cells, NK CD56<sup>bright</sup> cells, B cells, iDCs, and aDCs. High expression of *REG4* was negatively correlated with Tcm cell infiltration. In future research, we will focus on the impact of *REG4* expression on the immune environments of primary and metastatic CRC, which is also important for immunotherapy of CRC.

Previously, we discovered that expression of *REG4* was substantially linked with that of mucin-2 and mucin-5AC, and that it was greater in mucinous carcinoma, signet ring cell carcinoma, and intestinal metaplasia that produced mucins [28]. *REG4* was identified as a potential biomarker of mucinous ovarian cancer at both mRNA and protein levels[12,51]. Here, we also found that expression of *REG4* in mucinous adenocarcinoma was greater than that in other histological subtypes at both at the mRNA and protein levels. These findings could account for *REG4* protein overexpression in colorectal mucosa and poorly differentiated, signet ring cell carcinoma and undifferentiated carcinoma. *REG4* expression was positively correlated with lymph node metastasis, TNM staging, poorly differentiated CRC, and had a worse prognosis, in agreement with other studies[12,28,46-49]. The opposite was the case for *REG4* mRNA, suggesting that aberrant *REG4* protein expression might indicate aggressiveness and prognosis of CRC. In CRCs with reduced stroma compared to those with high stroma, *REG4* protein expression was considerably greater[52]. Previously, we performed *REG4* immunostaining on the same tissue microarrays of CRC (Supplementary Table 1), and found similar results. Therefore, the discrepancies about *REG4* mRNA and protein expression might be largely attributable to a complex process from transcription to translation and different methodologies. Serologically, the preoperative *REG4* protein level was higher in CRC than in a healthy population and postoperative patients with cancer, in agreement with the finding that serum *REG4* Levels were greater in pancreatic ductal adenocarcinoma than in chronic pancreatitis[53].

Bishnupuri *et al*[54] found that the transcriptional activator of D-type cyclins, CD44 intracytoplasmic domain, was released after *REG4* connected with transmembrane CD44 and activated  $\gamma$ -secretase to promote proliferation and stemness of colorectal and pancreatic cancer cells. We also found that *REG4* overexpression and rh*REG4* treatment promoted proliferation, antiapoptosis, and migration and invasion of CRC cells. Exposure to anti-*REG4* antibody inhibited the effects of *REG4* overexpression on the phenotypes of CRC cells, which is consistent with our earlier result in ovarian cancer cells[12]. We also found that *REG4* overexpression resulted in resistance of ovarian cancer cells to DDP or taxol by activating the PI3K-Akt-mTOR signaling pathway[55]. As a mutant KRAS-induced factor, *REG4* increased cancer stem cell characteristics *via* Wnt/ $\beta$ -catenin signaling[56]. Jin *et al*[57] showed that *REG4* increased the resistance of gastric cancer cells to 5-FU by stimulating the mitogen-activated protein kinase-ERK-Bim signaling pathway. Here, we found that expression of *REG4*, EGFR-Tyr992, -Tyr1068, -Tyr1148 and -Tyr1173, p-PI3K, p-Akt, NF- $\kappa$ B, p-NF- $\kappa$ B, Bcl-2, and Bcl-x/L was higher in DLD-1 cells treated with rh*REG4* or transfected with *REG4*-overexpressing plasmid than in parental cells. Anti-*REG4* antibody blocked the effects of *REG4* overexpression, indicating that *REG4* promoted the aggressive phenotypes by an EGFR-PI3K-Akt-NF- $\kappa$ B signaling pathway, in line with other studies[7,8,40]. However, the lack of effect of NSP-*REG4* on aggressive phenotypes and related signaling proteins suggested that *REG4* only functioned in





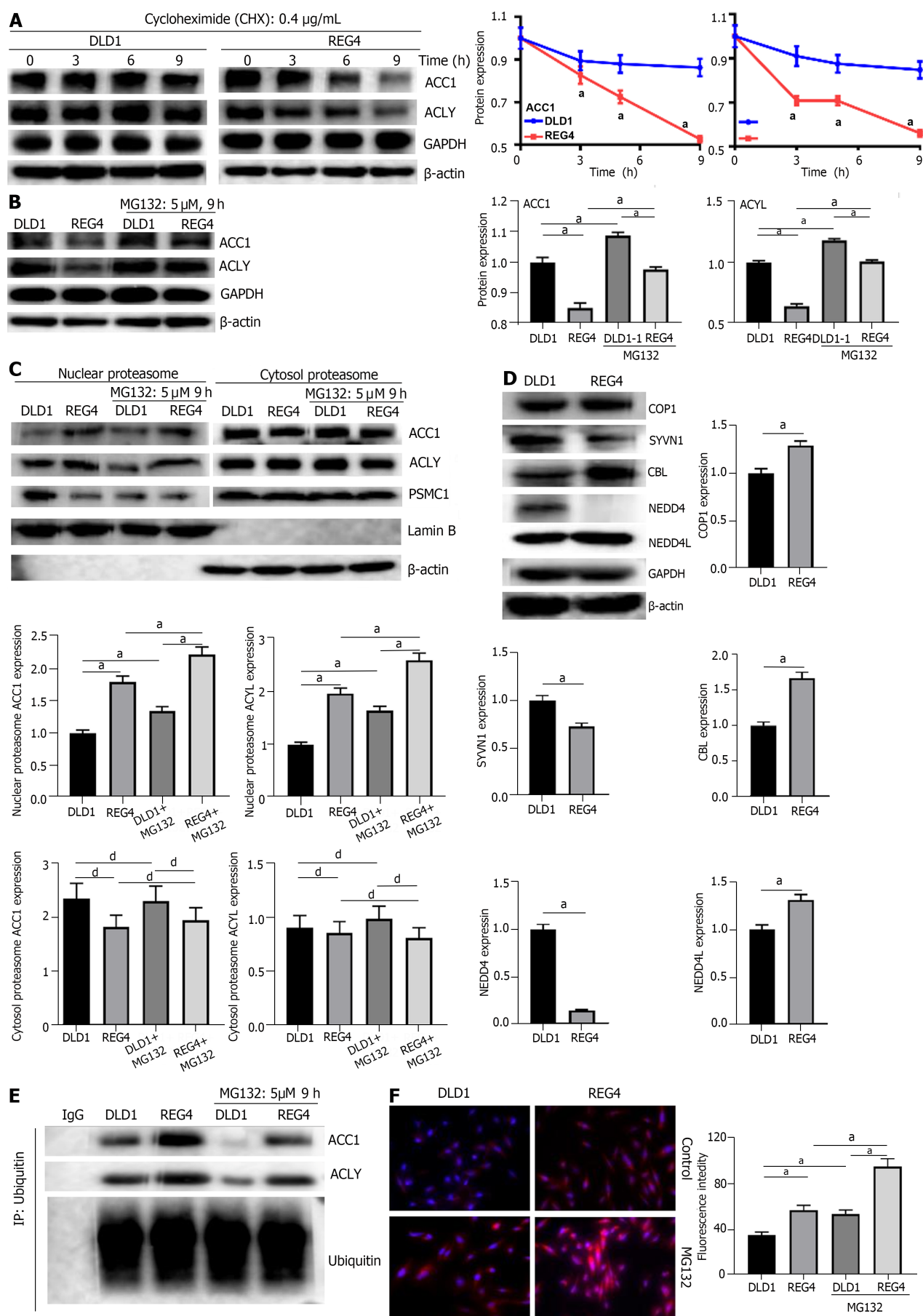


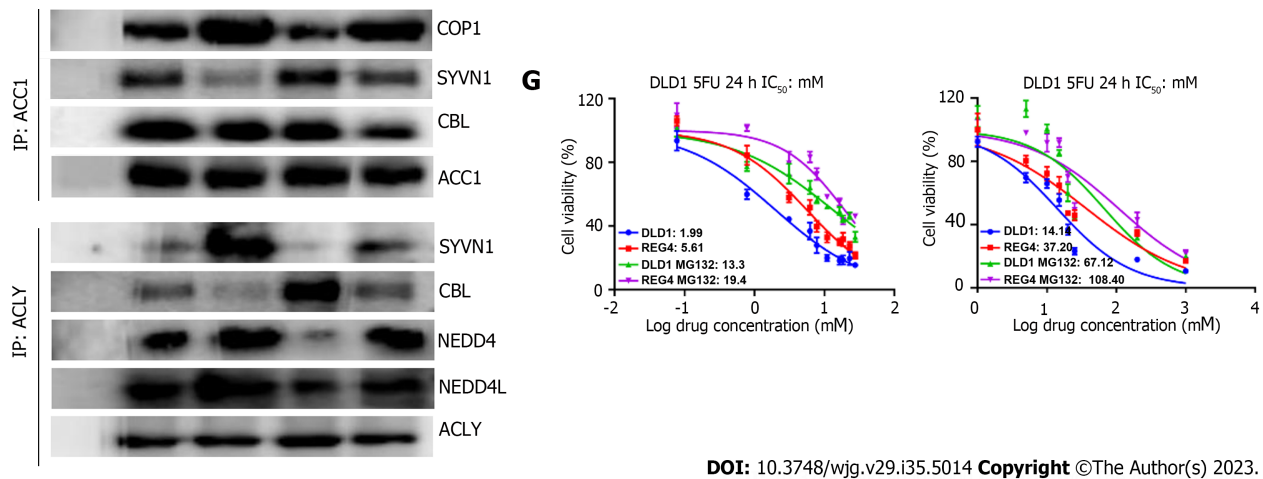
DOI: 10.3748/wjg.v29.i35.5104 Copyright ©The Author(s) 2023.

**Figure 3** Full-length regenerating gene 4 weakened the transcription of acetyl-CoA carboxylase 1 and ATP-citrate lyase. A: DLD-1 cells and full-length-regenerating gene 4 (FL-REG4) transfectants were treated with suberoylanilide hydroxamic acid (SAHA) or small interfering RNA against histone deacetylase (siHDAC), and analyzed by chromatin immunoprecipitation; B: DLD-1 cells and FL-REG4 transfectants were treated with SAHA, or siHDAC, and analyzed by co-immunoprecipitation assay using anti-acetyl (AC)-acetyl-histone 3-AC-histone 4, anti-HDAC, anti-sterol-regulatory element binding protein 1 or anti-inhibitor of growth protein 5 antibody; C: DLD-1 cells and FL-REG4 transfectants were treated with SAHA or siHDAC, and analyzed by quantitative reverse transcription polymerase chain reaction; D: DLD-1 cells and FL-REG4 transfectants were treated with SAHA or siHDAC, and analyzed by Nile red staining; E: DLD-1 cells and FL-REG4 transfectants were treated with SAHA or siHDAC, and analyzed by half maximal inhibitory concentration assay of 5-fluorouracil and cisplatin. <sup>a</sup>*P* < 0.001; <sup>b</sup>*P* < 0.01. REG4: Regenerating gene 4; SAHA: Suberoylanilide hydroxamic acid; siHDAC: Small interfering RNA against histone deacetylase; IgG: Immunoglobulin G; ND: No DNA; ACC1: Acetyl-CoA carboxylase 1; ACCY: ATP-citrate lyase; AC-H3: Acetyl-acetyl-histone 3; H4: Histone 4; SREBP1: Sterol-regulatory element binding protein 1; ING5: Inhibitor of growth protein 5; Ctr: Control DLD-1 cells; PC: Positive control.

CRC cells in an autocrine or paracrine manner. These findings demonstrated that *REG4* might be a potential molecular target for gene therapy of CRC.

DLD-1 cells developed resistance to 5-FU and DDP as a result of *REG4* overexpression. 5-FU- or DDP-resistant DLD-1 cells showed *REG4* overexpression, suggesting a role for *REG4* in chemoresistance, in line with previous studies[22,55]. The chemoresistance of CRC cells was produced by lysophosphatidylcholine acyltransferase 2-mediated lipid droplet formation[58], which was also aided by prothymosin  $\alpha$ [59], and metastasis-associated in colon cancer[60] through SREBP-1- and fatty acid synthase-mediated and lipogenesis, respectively. Crucial enzymes for de novo fatty acid synthesis are ACC1 and ACLY, which are closely linked to chemoresistance[61]. In the liver and peritoneal tissues, lipid droplet assembly is mediated by adipocyte differentiation-related protein, CIDE, ACAT1, perilipin 5 and TIP47[62-66]. *REG4*-mediated lipid droplet formation might be closely linked to the upregulated expression of ACAT1, perilipin 5 and TIP47 in *REG4* transfectants, but not with de novo lipogenesis, as demonstrated by the downregulated expression of ACC1 and ACLY. *REG4*-mediated lipid droplet formation might account for *REG4*-induced resistance to 5-FU and DDP, which can be reversed by ACC1 or ACLY inhibitor, but deteriorated by high glucose exposure. In combination with these discoveries, we hypothesized that *REG4* may play an important role in chemoresistance, not through de novo lipogenesis, but by lipid droplet assembly, and might be used as a potential target for reversal of chemoresistance in CRC. According to the increased IC<sub>50</sub> of DLD-1 cells after high glucose treatment, we speculate that high glucose treatment provides more glucose and increases the de novo synthesis of lipid droplets, thereby mediating the drug resistance of CRC cells.





**Figure 4** Full-length regenerating gene 4 destabilized acetyl-CoA carboxylase 1 and ATP-citrate lyase proteins via proteasomal degradation. A: DLD-1 cells and full-length-regenerating gene 4 (FL-REG4) transfectants were treated with cycloheximide (0.4  $\mu$ g/mL), followed by western blotting to detect acetyl-CoA carboxylase 1 (ACC1) and ATP-citrate lyase (ACLY) expression; B: DLD-1 cells and FL-REG4 transfectants were treated with MG132 (5  $\mu$ M, 9 h), followed by western blotting to detect ACC1 and ACLY expression; C: DLD-1 cells, FL-REG4 transfectants, and MG132-treated DLD-1 cells and FL-REG4 transfectants were subjected to proteasomal extract and western blotting to detect ACC1 and ACLY expression; D: Ubiquitin transferases (COP1 E3 ubiquitin ligase, synoviolin 1, Cbl proto-oncogene, NEDD4 like E3 ubiquitin protein ligase) were detected by western blotting in DLD-1 cells and FL-REG4 transfectants; E: After co-immunoprecipitation, western blotting was performed in DLD-1 cells and FL-REG4 transfectants, with or without MG132 treatment; F: Nile red staining of DLD-1 cells, FL-REG4 transfectants, and MG132-treated DLD-1 cells and FL-REG4 transfectants; G: Tetrazolium salt assay was performed on DLD-1 cells, FL-REG4 transfectants and MG132-treated DLD-1 cells and FL-REG4 transfectants to detect half maximal inhibitory concentration of 5-fluorouracil and cisplatin. \* $P < 0.001$ ; <sup>o</sup>No significance. REG4: Regenerating gene 4; ACC1: Acetyl-CoA carboxylase 1; ACLY: ATP-citrate lyase; COP1: COP1 E3 ubiquitin ligase; SYVN1: Synoviolin 1; CBL: Cbl proto-oncogene; NEDD4L: NEDD4 like E3 ubiquitin protein ligase; IgG: Immunoglobulin G.

We found that *REG4*-related signaling pathways included chemokine activity, taste receptors, protein-DNA and DNA packing complexes for transcription repression and activation, nucleosomes and chromatin, generation of second messenger molecules, HDAC and histone acetyltransferase for epigenetic regulation, and sugar metabolism. Therefore, we investigated the regulatory effects of *REG4* on the transcription of *ACC1* and *ACLY*. *REG4* decreased expression of AC-H3, AC-H4, ING5, HDAC and SREBP1. *REG4* also weakened the interaction of the five proteins with the promoters of *ACC1* or *ACLY*, or complex formation of the five proteins, and mRNA expression of *ACC1* or *ACLY*. The inhibitory effect of FL-*REG4* transcription can be enhanced by low concentration SAHA (HDAC inhibitor) treatment or transfection with siHDAC. We speculated that HDAC hypoexpression or inactivation might increase the chemosensitivity of CRC cells by inhibiting the de novo lipogenesis, which suggests a potential clinical application for the antitumor drug SAHA. The decrease in lipid droplet formation and increase in chemotherapy sensitivity of DLD-1 cells after SAHA or siHDAC treatment indirectly confirm this view. We hypothesized that *REG4* inhibited the transcription of *ACC1* and *ACLY* by disassociating the complex formation of AC-H3-AC-H4-ING5-HDAC-SREBP1 in their promoters and releasing the combination of *ACC1* and *ACLY* promoters and complex *via* HDAC-mediated deacetylation.

To date, there has been no research about the effects of *REG4* on metabolic reprogramming, histone modification, and microenvironmental stress, and the interaction between *REG4* and metabolic or epigenetic pathways in CRC. With regard to drug resistance, Ying *et al*[67] found that upregulation of *REG4* mRNA was closely linked to the intrinsic drug resistance of gastric cancer cells to 5-FU. All 14 *REG4*-positive patients with gastric cancer showed no change or disease progression when treated with a combination of low-dose 5-FU and DDP[1]. In gastric cancer cells, *REG4* enhanced the resistance to 5-FU through the MAPK-ERK-Bim pathway[1]. Anti-*REG4* antibody significantly inhibited proliferation and chemosensitivity of gastric cancer cells to 5-FU, and *REG4* silencing caused the loss of stemness properties[68,69]. In ovarian cancer cells, *REG4* overexpression or rh*REG4* treatment promoted proliferation, G<sub>2</sub>/S progression, antiapoptosis, migration, invasion, and DDP and paclitaxel[12,57]. In future research, we will also explore the potential interaction between *REG4* and metabolic or epigenetic pathways in CRC, which provides a new method for the early diagnosis and targeted therapy of CRC.

Finally, we also analyzed the modulatory effects of *REG4* on the protein stability of *ACC1* and *ACLY*. Firstly, we treated DLD-1 cells and *REG4* transfectants with CHX, and found that *REG4* destabilized *ACC1* and *ACLY*. *REG4* promoted the recruitment of *ACC1* and *ACLY* to the nuclear proteasome for ubiquitylation-mediated degradation, during which *ACLY* bound to NEDD4, SYVN1 and NEDD4L, and *ACC1* bound to COP1. Overexpression of *REG4* mediated lipid droplet formation and chemoresistance in DLD-1 cells. These results suggested that *REG4* facilitated proteasomal degradation of *ACC1* and *ACLY* to suppress de novo lipogenesis. We also speculate that pretreatment of proteasome inhibitor MG132 in DLD-1 cells can increase expression of *ACC1* and *ACLY* by reducing ubiquitination of *ACC1* and *ACLY*, thus increasing formation of lipid droplets and leading to chemoresistance. In the future, we will explore the potential role of *REG4* from metabolic reprogramming and epigenetic regulation.

## CONCLUSION

*REG4* protein expression was decreased in CRC and positively linked with the degree of invasion, TNM stage, and dedifferentiation, but the converse was the case for *REG4* mRNA expression. *REG4* aggravated aggressive phenotypes in an autocrine or paracrine manner *via* the EGFR-PI3K-Akt-NF- $\kappa$ B signaling pathway. *REG4* may be involved in chemoresistance, not through de novo lipogenesis, but by lipid droplet assembly. *REG4* inhibited the transcription of ACC1 or ACLY by disassociating the complex formation of AC-H3-AC-H4-ING5-histone deacetylase-SREBP1 in their promoters and induced the proteasomal degradation of ACC1 and ACLY proteins. Pretreatment with high glucose might induce chemoresistance of CRC cells, which should be emphasized in clinical practice. SAHA can reverse the chemoresistance of CRC and provide a potential direction for research of DDP and 5-FU resistance of CRC. Finally, *REG4* may be used as a reliable diagnostic marker for the prognosis, aggressiveness and carcinogenesis of CRC and is a potential molecular target.

## ARTICLE HIGHLIGHTS

### Research background

Regulating gene 4 (*REG4*) has been proved to be carcinogenic in some cancers, but its manifestation and possible carcinogenic mechanism in colorectal cancer (CRC) have not yet been elucidated. Our previous study found that the drug resistance characteristics of CRC cells may be related to their fat metabolism.

### Research motivation

With the aging of the world population, the incidence of CRC is increasing. For the treatment of CRC, chemoresistance has always been an urgent problem to be solved.

### Research objectives

This study aimed to explore the role of *REG4* in CRC and its association with lipid droplet formation, and the molecular mechanisms involved.

### Research methods

We conducted a meta-analysis and bioinformatics and pathological analysis of *REG4* expression in CRC. The effects of *REG4* on the phenotypes and related proteins were also investigated in CRC cells.

### Research results

Compared to normal mucosa, *REG4* mRNA expression was high in CRC, but protein expression was opposite. *REG4*-related genes included epigenetic regulation, transcription repression, sugar metabolism and transfer. *REG4* exposure or overexpression promoted proliferation, antiapoptosis, migration and invasion of DLD-1 cells in an autocrine or paracrine manner by activating the epidermal growth factor receptor-phosphoinositide 3-kinase-Akt-nuclear factor- $\kappa$ B pathway. *REG4* was involved in chemoresistance not through de novo lipogenesis, but lipid droplet assembly, which was strengthened by high glucose treatment. *REG4* inhibited the transcription of acetyl-coA carboxylase 1 (ACC1) and ATP-citrate lyase (ACLY) by disassociating the complex formation of anti-acetyl (AC)-acetyl-histone 3-AC-histone 4-inhibitor of growth protein-5-si histone deacetylase-sterol-regulatory element binding protein 1 in their promoters and induced proteasomal degradation of ACC1 or ACLY.

### Research conclusions

*REG4* may be an indicator of drug resistance and metabolism of tumor cells. *REG4* might be a useful marker for colorectal carcinogenesis, as well as a potential gene therapy target.

### Research perspectives

This study provides new insights into a better understanding of the pathogenesis of CRC. *REG4* may be used as a novel therapeutic target. However, the regulatory mechanism needs to be further explored.

## FOOTNOTES

**Author contributions:** Zhang CY and Zheng HC designed the study; Zhang CY participated in data collection; Zheng HC wrote the paper; Zhang R, Zhang L, Wang ZM, Sun HZ and Cui ZG were responsible for revising the manuscript; All the listed authors have contributed and approved the final manuscript.

**Supported by** Natural Science Foundation of Hebei Province, No. 21377772D; No. H2022406034; National Natural Scientific Foundation of China, No. 81672700.

**Institutional review board statement:** The study was reviewed and approved by the Institutional Review Board at the First Affiliated



Hospital of Jinzhou Medical University.

**Informed consent statement:** All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

**Conflict-of-interest statement:** All the authors report no relevant conflicts of interest for this article.

**Data sharing statement:** No additional data are available.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <https://creativecommons.org/licenses/by-nc/4.0/>

**Country/Territory of origin:** China

**ORCID number:** Cong-Yu Zhang 0000-0002-2089-9653; Rui Zhang 0000-0003-0197-1098; Li Zhang 0009-0004-8547-1326; Zi-Mo Wang 0000-0002-4948-8612; Hong-Zhi Sun 0009-0001-5259-1530; Zheng-Guo Cui 0000-0002-9756-9150; Hua-Chuan Zheng 0000-0003-0965-6818.

**S-Editor:** Qu XL

**L-Editor:** A

**P-Editor:** Cai YX

## REFERENCES

- 1 **Zheng HC**, Xue H, Zhang CY. *REG4* promotes the proliferation and anti-apoptosis of cancer. *Front Cell Dev Biol* 2022; **10**: 1012193 [PMID: 36172286 DOI: 10.3389/fcell.2022.1012193]
- 2 **Hu Y**, Pan C, Hu J, Zhang S. The role of Reg IV in colorectal cancer, as a potential therapeutic target. *Contemp Oncol (Pozn)* 2015; **19**: 261-264 [PMID: 26557771 DOI: 10.5114/wo.2015.54385]
- 3 **Zheng HC**, Sugawara A, Okamoto H, Takasawa S, Takahashi H, Masuda S, Takano Y. Expression profile of the REG gene family in colorectal carcinoma. *J Histochem Cytochem* 2011; **59**: 106-115 [PMID: 21339177 DOI: 10.1369/jhc.2010.956961]
- 4 **Wang H**, Hu L, Zang M, Zhang B, Duan Y, Fan Z, Li J, Su L, Yan M, Zhu Z, Liu B, Yang Q. *REG4* promotes peritoneal metastasis of gastric cancer through GPR37. *Oncotarget* 2016; **7**: 27874-27888 [PMID: 27036049 DOI: 10.18632/oncotarget.8442]
- 5 **Kawasaki Y**, Matsumura K, Miyamoto M, Tsuji S, Okuno M, Suda S, Hiyoshi M, Kitayama J, Akiyama T. *REG4* is a transcriptional target of GATA6 and is essential for colorectal tumorigenesis. *Sci Rep* 2015; **5**: 14291 [PMID: 26387746 DOI: 10.1038/srep14291]
- 6 **Naito Y**, Oue N, Hinoi T, Sakamoto N, Sentani K, Ohdan H, Yanagihara K, Sasaki H, Yasui W. Reg IV is a direct target of intestinal transcriptional factor CDX2 in gastric cancer. *PLoS One* 2012; **7**: e47545 [PMID: 23133598 DOI: 10.1371/journal.pone.0047545]
- 7 **Bishnupuri KS**, Sainathan SK, Bishnupuri K, Leahy DR, Luo Q, Anant S, Houchen CW, Dieckgraefe BK. *Reg4*-induced mitogenesis involves Akt-GSK3 $\beta$ -Catenin-TCF-4 signaling in human colorectal cancer. *Mol Carcinog* 2014; **53** Suppl 1: E169-E180 [PMID: 24151146 DOI: 10.1002/mc.22088]
- 8 **Bishnupuri KS**, Luo Q, Murmu N, Houchen CW, Anant S, Dieckgraefe BK. Reg IV activates the epidermal growth factor receptor/Akt/AP-1 signaling pathway in colon adenocarcinomas. *Gastroenterology* 2006; **130**: 137-149 [PMID: 16401477 DOI: 10.1053/j.gastro.2005.10.001]
- 9 **He XJ**, Jiang XT, Ma YY, Xia YJ, Wang HJ, Guan TP, Shao QS, Tao HQ. *REG4* contributes to the invasiveness of pancreatic cancer by upregulating MMP-7 and MMP-9. *Cancer Sci* 2012; **103**: 2082-2091 [PMID: 22957785 DOI: 10.1111/cas.12018]
- 10 **Ma X**, Wu D, Zhou S, Wan F, Liu H, Xu X, Zhao Y, Tang M. The pancreatic cancer secreted *REG4* promotes macrophage polarization to M2 through EGFR/AKT/CREB pathway. *Oncol Rep* 2016; **35**: 189-196 [PMID: 26531138 DOI: 10.3892/or.2015.4357]
- 11 **Mitani Y**, Oue N, Matsumura S, Yoshida K, Noguchi T, Ito M, Tanaka S, Kuniyasu H, Kamata N, Yasui W. Reg IV is a serum biomarker for gastric cancer patients and predicts response to 5-fluorouracil-based chemotherapy. *Oncogene* 2007; **26**: 4383-4393 [PMID: 17237819 DOI: 10.1038/sj.onc.1210215]
- 12 **Chen S**, Gou WF, Zhao S, Niu ZF, Zhao Y, Takano Y, Zheng HC. The role of the *REG4* gene and its encoding product in ovarian epithelial carcinoma. *BMC Cancer* 2015; **15**: 471 [PMID: 26077911 DOI: 10.1186/s12885-015-1435-2]
- 13 **Violette S**, Festor E, Pandrea-Vasile I, Mitchell V, Adida C, Dussault E, Lacorte JM, Chambaz J, Lacasa M, Lesuffleur T. Reg IV, a new member of the regenerating gene family, is overexpressed in colorectal carcinomas. *Int J Cancer* 2003; **103**: 185-193 [PMID: 12455032 DOI: 10.1002/ijc.10788]
- 14 **Granlund AV**, Beisvag V, Torp SH, Flatberg A, Kleiveland PM, Ostvik AE, Waldum HL, Sandvik AK. Activation of REG family proteins in colitis. *Scand J Gastroenterol* 2011; **46**: 1316-1323 [PMID: 21992413 DOI: 10.3109/00365521.2011.605463]
- 15 **Oue N**, Kuniyasu H, Noguchi T, Sentani K, Ito M, Tanaka S, Setoyama T, Sakakura C, Natsugoe S, Yasui W. Serum concentration of Reg IV in patients with colorectal cancer: overexpression and high serum levels of Reg IV are associated with liver metastasis. *Oncology* 2007; **72**: 371-380 [PMID: 18187959 DOI: 10.1159/000113147]
- 16 **Numata M**, Oshima T, Yoshihara K, Watanabe T, Tsuchida K, Tamagawa H, Yamamoto N, Shiozawa M, Morinaga S, Akaike M, Kunisaki C, Rino Y, Tanaka K, Masuda M, Imada T. Relationship between RegIV gene expression to outcomes in colorectal cancer. *J Surg Oncol* 2011; **104**: 205-209 [PMID: 21381041 DOI: 10.1002/jso.21906]
- 17 **He HL**, Lee YE, Shiue YL, Lee SW, Lin LC, Chen TJ, Wu TF, Hsing CH, Huang HY, Wang JY, Li CF. Overexpression of *REG4* confers an independent negative prognosticator in rectal cancers receiving concurrent chemoradiotherapy. *J Surg Oncol* 2014; **110**: 1002-1010 [PMID: 25155043 DOI: 10.1002/jso.23764]
- 18 **Guo Y**, Xu J, Li N, Gao F, Huang P. RegIV potentiates colorectal carcinoma cell migration and invasion via its CRD domain. *Cancer Genet*

- Cytogenet* 2010; **199**: 38-44 [PMID: 20417867 DOI: 10.1016/j.cancergencyto.2010.01.011]
- 19 **Rafa L**, Dessein AF, Devisme L, Buob D, Truant S, Porchet N, Huet G, Buisine MP, Lesuffleur T. *REG4* acts as a mitogenic, motility and pro-invasive factor for colon cancer cells. *Int J Oncol* 2010; **36**: 689-698 [PMID: 20126989 DOI: 10.3892/ijo.00000544]
  - 20 **Nanakin A**, Fukui H, Fujii S, Sekikawa A, Kanda N, Hisatsune H, Seno H, Konda Y, Fujimori T, Chiba T. Expression of the REG IV gene in ulcerative colitis. *Lab Invest* 2007; **87**: 304-314 [PMID: 17260007 DOI: 10.1038/Labinvest.3700507]
  - 21 **Kobunai T**, Watanabe T, Fukusato T. *REG4*, NEIL2, and BIRC5 gene expression correlates with gamma-radiation sensitivity in patients with rectal cancer receiving radiotherapy. *Anticancer Res* 2011; **31**: 4147-4153 [PMID: 22199273]
  - 22 **Gao L**, Wu X, Zhang L, Dai Y, Zhu Z, Zhi Y, Wang K. *REG4* is a Potential Biomarker for Radiochemotherapy Sensitivity in Colorectal Cancer. *Onco Targets Ther* 2021; **14**: 1605-1611 [PMID: 33688207 DOI: 10.2147/OTT.S296031]
  - 23 **Jou E**, Rodriguez-Rodriguez N, McKenzie ANJ. Emerging roles for IL-25 and IL-33 in colorectal cancer tumorigenesis. *Front Immunol* 2022; **13**: 981479 [PMID: 36263033 DOI: 10.3389/fimmu.2022.981479]
  - 24 **Droeser RA**, Iezzi G. IL-22-mediate Cross-talk between Tumor Cells and Immune Cells Associated with Favorable Prognosis in Human Colorectal Cancer. *J Cell Immunol* 2021; **3**: 118-121 [PMID: 34296212 DOI: 10.33696/immunology.3.087]
  - 25 **Djaldetti M**, Bessler H. Modulators affecting the immune dialogue between human immune and colon cancer cells. *World J Gastrointest Oncol* 2014; **6**: 129-138 [PMID: 24834143 DOI: 10.4251/wjgo.v6.i5.129]
  - 26 **Li Y**, Liu Y, Zhao N, Yang X, Li Y, Zhai F, Zang X, Cui W. Checkpoint regulator B7x is epigenetically regulated by HDAC3 and mediates resistance to HDAC inhibitors by reprogramming the tumor immune environment in colorectal cancer. *Cell Death Dis* 2020; **11**: 753 [PMID: 32934224 DOI: 10.1038/s41419-020-02968-y]
  - 27 **Jang TJ**. Progressive Increase of Regulatory T Cells and Decrease of CD8+ T Cells and CD8+ T Cells/Regulatory T Cells Ratio during Colorectal Cancer Development. *Korean J Pathol* 2013; **47**: 443-451 [PMID: 24255632 DOI: 10.4132/KoreanJPathol.2013.47.5.443]
  - 28 **Zheng HC**, Xu XY, Yu M, Takahashi H, Masuda S, Takano Y. The role of Reg IV gene and its encoding product in gastric carcinogenesis. *Hum Pathol* 2010; **41**: 59-69 [PMID: 19740514 DOI: 10.1016/j.humpath.2009.06.013]
  - 29 **Zheng SQ**, He J, Huang PL, Wang JH. Clinical significance of Reg IV protein expression in human colorectal cancer. *Shijie Huaren Xiaohua Zazhi* 2010; **18**: 3594-3598 [DOI: 10.11569/wjcd.v18.i33.3594]
  - 30 **Li XH**, Zheng Y, Zheng HC, Takahashi H, Yang XH, Masuda S, Takano Y. REG IV overexpression in an early stage of colorectal carcinogenesis: an immunohistochemical study. *Histol Histopathol* 2010; **25**: 473-484 [PMID: 20183800 DOI: 10.14670/HH-25.473]
  - 31 **Li FY**, Ren XB, Xu EP, Huang Q, Sheng HQ, Lv BJ, Lai MD. RegIV expression showing specificity to gastrointestinal tract and its potential role in diagnosing digestive tract neuroendocrine tumor. *J Zhejiang Univ Sci B* 2010; **11**: 258-266 [PMID: 20349522 DOI: 10.1631/jzus.B0900383]
  - 32 **Wang LN**, Hou SP, Liu HB, Sun XL, Zhang XJ, Zhang QQ. Expression of *REG4* and surviving and clinical significance in colorectal cancer. *Shiyong Yixue Zazhi* 2015; **31**: 3347-3350 [DOI: 10.1186/s13000-015-0253-9]
  - 33 **Wang GM**, Wang CX, Nie MN, Zhang LH, Diao WY. Expression of Reg IV in colon carcinoma and its relationship with angiogenesis. *J Med Forum* 2016; **37**: 7-9 [DOI: 10.3892/ijmm.00000007]
  - 34 **Oue N**, Mitani Y, Aung PP, Sakakura C, Takeshima Y, Kaneko M, Noguchi T, Nakayama H, Yasui W. Expression and localization of Reg IV in human neoplastic and non-neoplastic tissues: Reg IV expression is associated with intestinal and neuroendocrine differentiation in gastric adenocarcinoma. *J Pathol* 2005; **207**: 185-198 [PMID: 16086444 DOI: 10.1002/path.1827]
  - 35 **Sawada T**, Yashiro M, Sentani K, Oue N, Yasui W, Miyazaki K, Kai K, Fujita H, Nakamura K, Maeda K, Kakeji Y, Natsugoe S, Shirabe K, Nomura S, Shimada Y, Tomita N, Hirakawa K, Maehara Y. New molecular staging with G-factors (VEGF-C and Reg IV) by supplementing TNM classification in colorectal cancers. *Oncol Rep* 2013; **30**: 2609-2616 [PMID: 24101199 DOI: 10.3892/or.2013.2787]
  - 36 **Zhu X**, Han Y, Yuan C, Tu W, Qiu G, Lu S, Lu H, Peng Z, Zhou C. Overexpression of *Reg4*, alone or combined with MMP-7 overexpression, is predictive of poor prognosis in colorectal cancer. *Oncol Rep* 2015; **33**: 320-328 [PMID: 25338725 DOI: 10.3892/or.2014.3559]
  - 37 **Kaprio T**, Hagström J, Mustonen H, Koskensalo S, Andersson LC, Haglund C. *REG4* independently predicts better prognosis in non-mucinous colorectal cancer. *PLoS One* 2014; **9**: e109600 [PMID: 25295732 DOI: 10.1371/journal.pone.0109600]
  - 38 **Takehara A**, Eguchi H, Ohigashi H, Ishikawa O, Kasugai T, Hosokawa M, Katagiri T, Nakamura Y, Nakagawa H. Novel tumor marker *REG4* detected in serum of patients with resectable pancreatic cancer and feasibility for antibody therapy targeting *REG4*. *Cancer Sci* 2006; **97**: 1191-1197 [PMID: 16918991 DOI: 10.1111/j.1349-7006.2006.00297.x]
  - 39 **Liu CM**, Hsieh CL, He YC, Lo SJ, Liang JA, Hsieh TF, Jossan S, Chung LW, Hung MC, Sung SY. In vivo targeting of ADAM9 gene expression using lentivirus-delivered shRNA suppresses prostate cancer growth by regulating *REG4* dependent cell cycle progression. *PLoS One* 2013; **8**: e53795 [PMID: 23342005 DOI: 10.1371/journal.pone.0053795]
  - 40 **Bishnupuri KS**, Luo Q, Sainathan SK, Kikuchi K, Sureban SM, Sabarinathan M, Gross JH, Aden K, May R, Houchen CW, Anant S, Dieckgraefe BK. Reg IV regulates normal intestinal and colorectal cancer cell susceptibility to radiation-induced apoptosis. *Gastroenterology* 2010; **138**: 616-626, 626.e1 [PMID: 19900450 DOI: 10.1053/j.gastro.2009.10.050]
  - 41 **Zhang Y**, Lai M, Lv B, Gu X, Wang H, Zhu Y, Shao L, Wang G. Overexpression of Reg IV in colorectal adenoma. *Cancer Lett* 2003; **200**: 69-76 [PMID: 14550954 DOI: 10.1016/s0304-3835(03)00460-9]
  - 42 **Lasserre C**, Colnot C, Bréchet C, Poirier F. HIP/PAP gene, encoding a C-type lectin overexpressed in primary liver cancer, is expressed in nervous system as well as in intestine and pancreas of the postimplantation mouse embryo. *Am J Pathol* 1999; **154**: 1601-1610 [PMID: 10329612 DOI: 10.1016/S0002-9440(10)65413-2]
  - 43 **Lasserre C**, Christa L, Simon MT, Vernier P, Bréchet C. A novel gene (HIP) activated in human primary liver cancer. *Cancer Res* 1992; **52**: 5089-5095 [PMID: 1325291]
  - 44 **Kimura N**, Yonekura H, Okamoto H, Nagura H. Expression of human regenerating gene mRNA and its product in normal and neoplastic human pancreas. *Cancer* 1992; **70**: 1857-1863 [PMID: 1525759 DOI: 10.1002/1097-0142(19921001)70:7<1857::aid-cnrc2820700708>3.0.co;2-8]
  - 45 **Ohara S**, Oue N, Matsubara A, Mita K, Hasegawa Y, Hayashi T, Usui T, Amatya VJ, Takeshima Y, Kuniyasu H, Yasui W. Reg IV is an independent prognostic factor for relapse in patients with clinically localized prostate cancer. *Cancer Sci* 2008; **99**: 1570-1577 [PMID: 18754868 DOI: 10.1111/j.1349-7006.2008.00846.x]
  - 46 **Takayama R**, Nakagawa H, Sawaki A, Mizuno N, Kawai H, Tajika M, Yatabe Y, Matsuo K, Uehara R, Ono K, Nakamura Y, Yamao K. Serum tumor antigen *REG4* as a diagnostic biomarker in pancreatic ductal adenocarcinoma. *J Gastroenterol* 2010; **45**: 52-59 [PMID: 19789838 DOI: 10.1007/s00535-009-0114-y]

- 47 Wang Q, Deng J, Yuan J, Wang L, Zhao Z, He S, Zhang Y, Tu Y. Oncogenic reg IV is a novel prognostic marker for glioma patient survival. *Diagn Pathol* 2012; 7: 69 [PMID: 22713481 DOI: 10.1186/1746-1596-7-69]
- 48 Tamura H, Ohtsuka M, Washiro M, Kimura F, Shimizu H, Yoshidome H, Kato A, Seki N, Miyazaki M. Reg IV expression and clinicopathologic features of gallbladder carcinoma. *Hum Pathol* 2009; 40: 1686-1692 [PMID: 19716164 DOI: 10.1016/j.humpath.2009.06.001]
- 49 Hayashi T, Matsubara A, Ohara S, Mita K, Hasegawa Y, Usui T, Arihiro K, Norimura S, Sentani K, Oue N, Yasui W. Immunohistochemical analysis of Reg IV in urogenital organs: Frequent expression of Reg IV in prostate cancer and potential utility as serum tumor marker. *Oncol Rep* 2009; 21: 95-100 [PMID: 19082448]
- 50 Derakhshani A, Hashemzadeh S, Asadzadeh Z, Shadbad MA, Rasibonab F, Safarpour H, Jafarlou V, Solimando AG, Racanelli V, Singh PK, Najafi S, Javadrashid D, Brunetti O, Silvestris N, Baradaran B. Cytotoxic T-Lymphocyte Antigen-4 in Colorectal Cancer: Another Therapeutic Side of Capecitabine. *Cancers (Basel)* 2021; 13 [PMID: 34067631 DOI: 10.3390/cancers13102414]
- 51 Lehtinen L, Vesterkvist P, Roering P, Korpela T, Hattara L, Kaipio K, Mpindi JP, Hynninen J, Auranen A, Davidson B, Haglund C, Iljin K, Grenman S, Siitari H, Carpen O. REG4 Is Highly Expressed in Mucinous Ovarian Cancer: A Potential Novel Serum Biomarker. *PLoS One* 2016; 11: e0151590 [PMID: 26981633 DOI: 10.1371/journal.pone.0151590]
- 52 Kang G, Oh I, Pyo J, Kang D, Son B. Clinicopathological Significance and Prognostic Implications of REG4 Immunohistochemical Expression in Colorectal Cancer. *Medicina (Kaunas)* 2021; 57 [PMID: 34577861 DOI: 10.3390/medicina57090938]
- 53 Saukkonen K, Hagström J, Mustonen H, Lehtinen L, Carpen O, Andersson LC, Seppänen H, Haglund C. Prognostic and diagnostic value of REG4 serum and tissue expression in pancreatic ductal adenocarcinoma. *Tumour Biol* 2018; 40: 1010428318761494 [PMID: 29542402 DOI: 10.1177/1010428318761494]
- 54 Bishnupuri KS, Sainathan SK, Ciorba MA, Houchen CW, Dieckgraefe BK. Reg4 Interacts with CD44 to Regulate Proliferation and Stemness of Colorectal and Pancreatic Cancer Cells. *Mol Cancer Res* 2022; 20: 387-399 [PMID: 34753802 DOI: 10.1158/1541-7786.MCR-21-0224]
- 55 Xiang LW, Xue H, Ha MW, Yu DY, Xiao LJ, Zheng HC. The effects of REG4 expression on chemoresistance of ovarian cancer. *J Obstet Gynaecol* 2022; 42: 3149-3157 [PMID: 35929918 DOI: 10.1080/01443615.2022.2106834]
- 56 Hwang JH, Yoon J, Cho YH, Cha PH, Park JC, Choi KY. A mutant KRAS-induced factor REG4 promotes cancer stem cell properties via Wnt/β-catenin signaling. *Int J Cancer* 2020; 146: 2877-2890 [PMID: 31605540 DOI: 10.1002/ijc.32728]
- 57 Jin J, Lv H, Wu J, Li D, Chen K, Zhang F, Han J, Feng J, Zhang N, Yu H, Su D, Ying L. Regenerating Family Member 4 (Reg4) Enhances 5-Fluorouracil Resistance of Gastric Cancer Through Activating MAPK/Erk/Bim Signaling Pathway. *Med Sci Monit* 2017; 23: 3715-3721 [PMID: 28759561 DOI: 10.12659/msm.903134]
- 58 Cotte AK, Aires V, Fredon M, Limagne E, Derangère V, Thibaudin M, Humblin E, Scagliarini A, de Barros JP, Hillon P, Ghiringhelli F, Delmas D. Lysophosphatidylcholine acyltransferase 2-mediated lipid droplet production supports colorectal cancer chemoresistance. *Nat Commun* 2018; 9: 322 [PMID: 29358673 DOI: 10.1038/s41467-017-02732-5]
- 59 Jin L, Zhu LY, Pan YL, Fu HQ, Zhang J. Prothymosin α promotes colorectal carcinoma chemoresistance through inducing lipid droplet accumulation. *Mitochondrion* 2021; 59: 123-134 [PMID: 33872798 DOI: 10.1016/j.mito.2021.04.001]
- 60 Duan J, Chen L, Zhou M, Zhang J, Sun L, Huang N, Bin J, Liao Y, Liao W. MACC1 decreases the chemosensitivity of gastric cancer cells to oxaliplatin by regulating FASN expression. *Oncol Rep* 2017; 37: 2583-2592 [PMID: 28339092 DOI: 10.3892/or.2017.5519]
- 61 Sur S, Nakanishi H, Flaveny C, Ippolito JE, McHowat J, Ford DA, Ray RB. Inhibition of the key metabolic pathways, glycolysis and lipogenesis, of oral cancer by bitter melon extract. *Cell Commun Signal* 2019; 17: 131 [PMID: 31638999 DOI: 10.1186/s12964-019-0447-y]
- 62 Fan H, Diao H, Lu Y, Xie J, Cheng X. The relation between serum adipose differentiation-related protein and non-alcoholic fatty liver disease in type 2 diabetes mellitus. *Ther Adv Endocrinol Metab* 2020; 11: 2042018820969025 [PMID: 33194172 DOI: 10.1177/2042018820969025]
- 63 Kasano-Camones CI, Takizawa M, Iwasaki W, Sasaki S, Hamada M, Morimoto A, Sakaguchi M, Gonzalez FJ, Inoue Y. Synergistic regulation of hepatic Fsp27b expression by HNF4α and CREBH. *Biochem Biophys Res Commun* 2020; 530: 432-439 [PMID: 32553626 DOI: 10.1016/j.bbrc.2020.05.070]
- 64 Ayyagari V, Li M, Pasman Z, Wang X, Louis S, Diaz-Sylvester P, Groesch K, Wilson T, Brard L. Assessment of the diagnostic and prognostic relevance of ACAT1 and CE levels in plasma, peritoneal fluid and tumor tissue of epithelial ovarian cancer patients - a pilot study. *BMC Cancer* 2022; 22: 387 [PMID: 35399074 DOI: 10.1186/s12885-022-09476-6]
- 65 Ethem İ, Hacıoğlu C. Effects of perilipin-5 on lipid metabolism and high-sensitivity cardiac troponin I. *Rev Assoc Med Bras (1992)* 2022; 68: 1011-1016 [PMID: 36134829 DOI: 10.1590/1806-9282.20211377]
- 66 Nose F, Yamaguchi T, Kato R, Aiuchi T, Obama T, Hara S, Yamamoto M, Itabe H. Crucial role of perilipin-3 (TIP47) in formation of lipid droplets and PGE2 production in HL-60-derived neutrophils. *PLoS One* 2013; 8: e71542 [PMID: 23936516 DOI: 10.1371/journal.pone.0071542]
- 67 Ying LS, Yu JL, Lu XX, Ling ZQ. Enhanced RegIV expression predicts the intrinsic 5-fluorouracil (5-FU) resistance in advanced gastric cancer. *Dig Dis Sci* 2013; 58: 414-422 [PMID: 23010741 DOI: 10.1007/s10620-012-2381-3]
- 68 Zhang XQ, Yu LT, Du P, Yin TQ, Zhang ZY, Xu Y, Li X, Li YJ, Wang M, Luo C. Single-chain Antibody Against Reg4 Suppresses Gastric Cancer Cell Growth and Enhances 5-FU-induced Cell Death in vitro. *Anticancer Agents Med Chem* 2019; 19: 610-619 [PMID: 30465515 DOI: 10.2174/1871520619666181122104720]
- 69 Zhou W, Sun M, Wang DL, Wang Y, Jin F, Zhang YY, Yang L, Wu XL, Wu YZ. Silencing of RegIV by shRNA causes the loss of stemness properties of cancer stem cells in MKN45 gastric cancer cells. *Oncol Rep* 2013; 30: 2685-2690 [PMID: 24064664 DOI: 10.3892/or.2013.2745]



Published by **Baishideng Publishing Group Inc**  
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA

**Telephone:** +1-925-3991568

**E-mail:** [bpgoffice@wjgnet.com](mailto:bpgoffice@wjgnet.com)

**Help Desk:** <https://www.f6publishing.com/helpdesk>

<https://www.wjgnet.com>

