

Basic Study

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

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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**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- κ 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

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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- κ 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.

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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 β / β -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) α , EGF, basic fibroblast growth factor, and hepatocyte growth factor in colon cancer cells, and then promoted cell proliferation and resistance to H₂O₂-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 γ -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- α , 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⁺ T cells and CD8⁺ 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 μ g/mL streptomycin in a humidified atmosphere of 5% CO₂ 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 μ L MTT reagent (5 mg/mL) was added to each well. After incubation for 4 h, 150 μ 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 μ L) was gently mixed with FITC-labeled Annexin-V (5 μ L) and PI (5 μ 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 10⁵ per well) were plated in six-well culture plates. When the cells reached 75% confluence, they were scraped with a pip, 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 1.3×10^5 cells per 200 μ 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'-AATCGCGGGCCGTTCTC-3', melting temperature (TM): 57.9 °C; 5'-CGACGAACCCCGCAAAATC-3', TM: 55.4 °C, -43 bp to + 81 bp) primers or ACC1 (5'-GCCCGAATGGCAGATCC-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 μ 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 μ 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- μ 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-GTCCTTTACTCG-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'-TGGAAGATGGTGTATGGGATT-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). H₂O₂ 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, 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 χ^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^{bright} 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 rh*REG4*, 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 rh*REG4* 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 rh*REG4* treatment enhanced the expression of *REG4*, EGFR-Tyr992, -Tyr1068, -Tyr1148, -Tyr1173, phosphorylated phosphoinositide 3-kinase (p-PI3K), p-Akt, nuclear factor (NF)- κ B, p-NF- κ 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

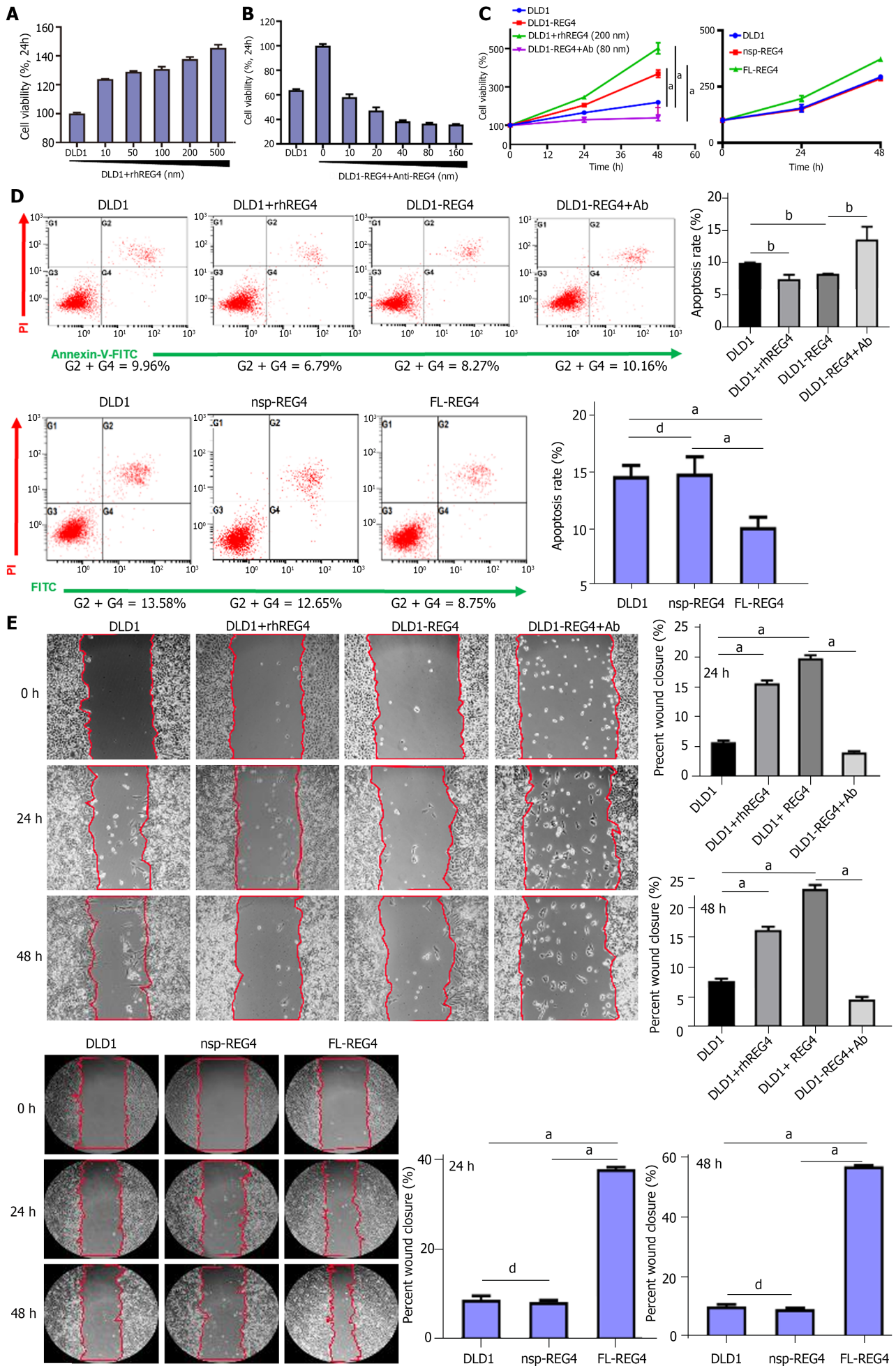
Rh*REG4* treatment and FL-*REG4* overexpression caused DLD-1 cells to become resistant to DDP and 5-FU ($P < 0.05$) (Figure 2A), while anti-*REG4* antibody reversed the chemoresistance of *REG4*-overexpressing DLD-1 cells ($P < 0.05$) (Figure 2A). rh*REG4* treatment and FL-*REG4* increased the formation 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 rh*REG4*, 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 ($P < 0.05$).

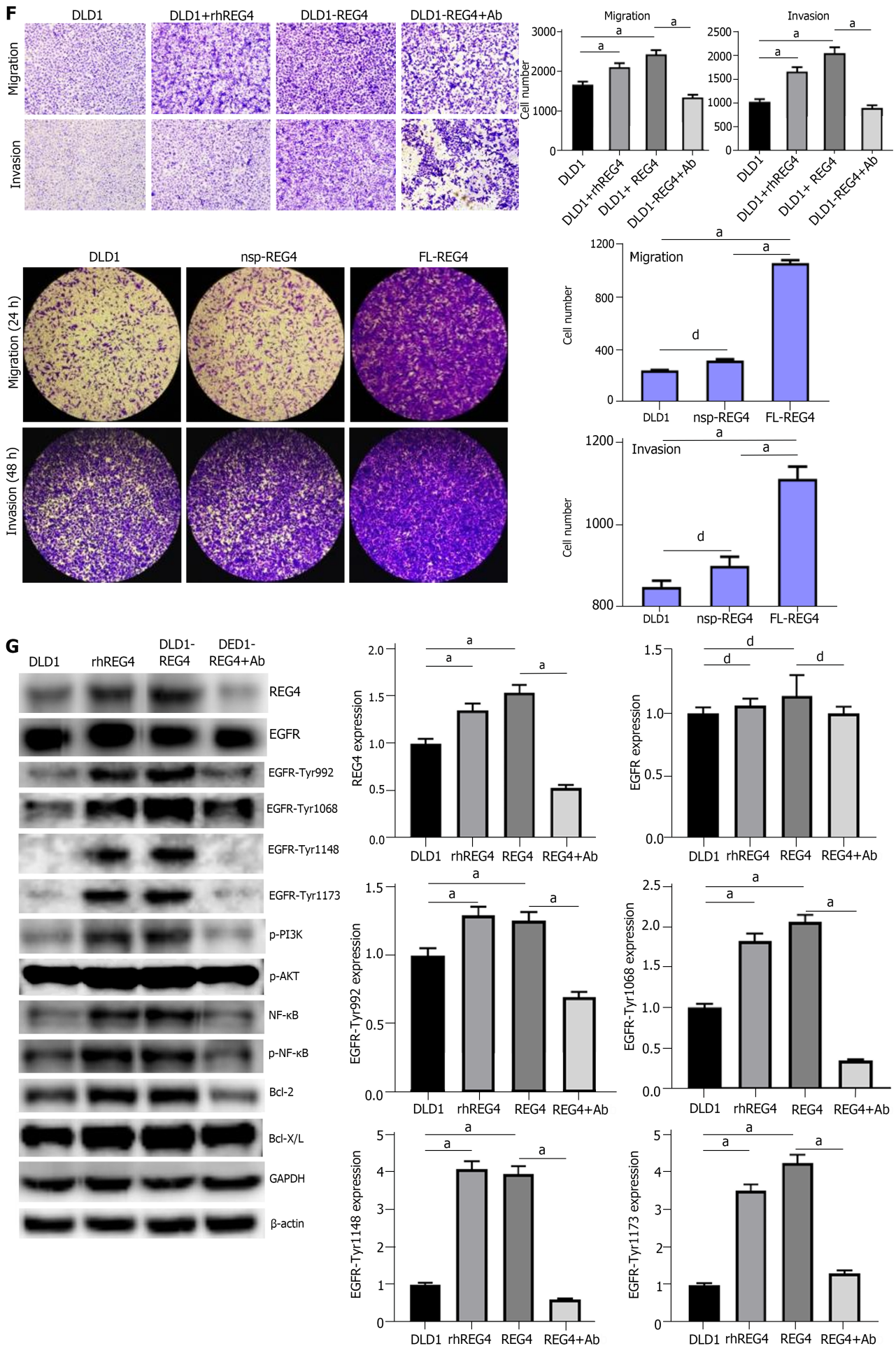
REG4 weakened transcription of ACLY and ACC1 via disassociating AC-H3-AC-H4-HDAC-ING5-SREBP1 complex

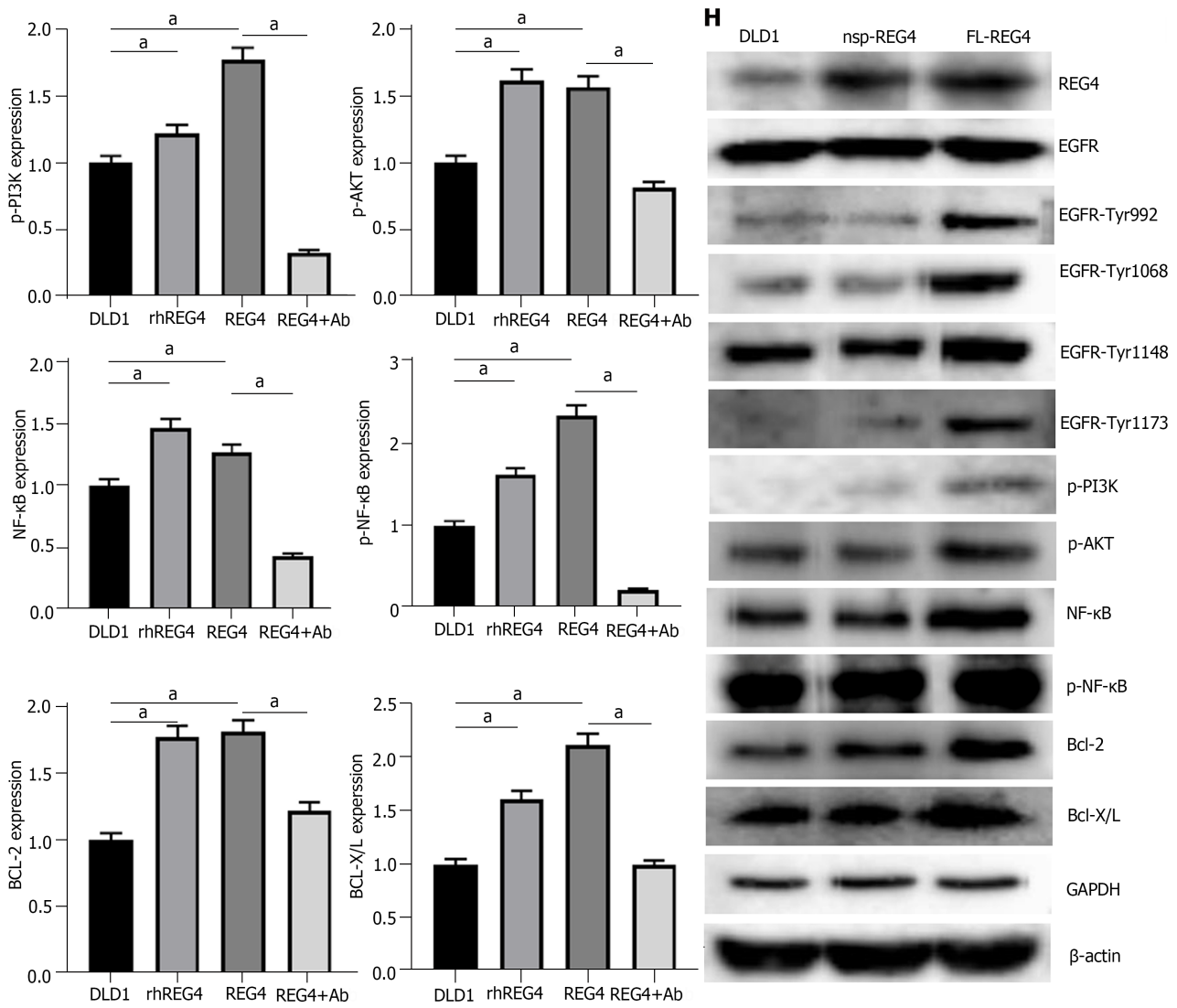
In DLD-1 cells, FL-*REG4* overexpression weakened the interaction of AC-H3, AC-H4, HDAC, SREBP1 and ING5 proteins with the ACLY or ACC1 promoter, according to ChIP ($P < 0.05$) (Figure 3A). FL-*REG4* overexpression also weakened the interaction of the five proteins ($P < 0.05$) (Figure 3B). Overexpression 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 (IC₅₀) of cells for 5-FU and DDP ($P < 0.05$) (Figure 3E). After SAHA (siHDAC inhibitor) treatment (2 μ 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 μ M,







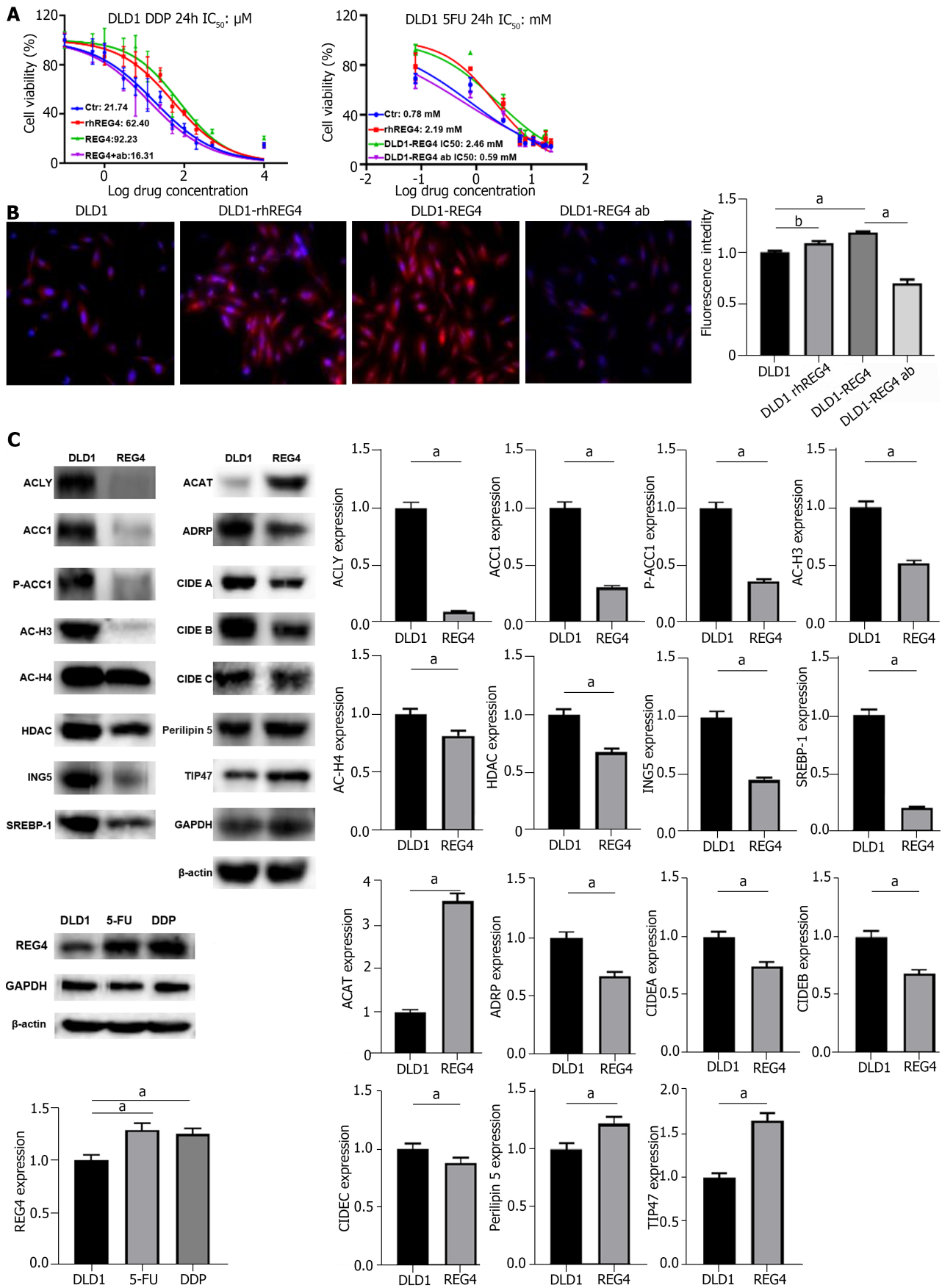
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Figure 1 Effects of full-length regenerating gene 4 and nonsignal peptide regenerating gene 4 on phenotypes of DLD-1 cells. A: DLD-1 cells were incubated with increasing doses of recombinant human regenerating gene 4 (*REG4*) (*rhREG4*) (0-500 nmol/L) 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. ^a*P* < 0.001; ^b*P* < 0.01; ^cNo 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 μM, 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 G₁/S transition[38,39]. Anti-*REG4*



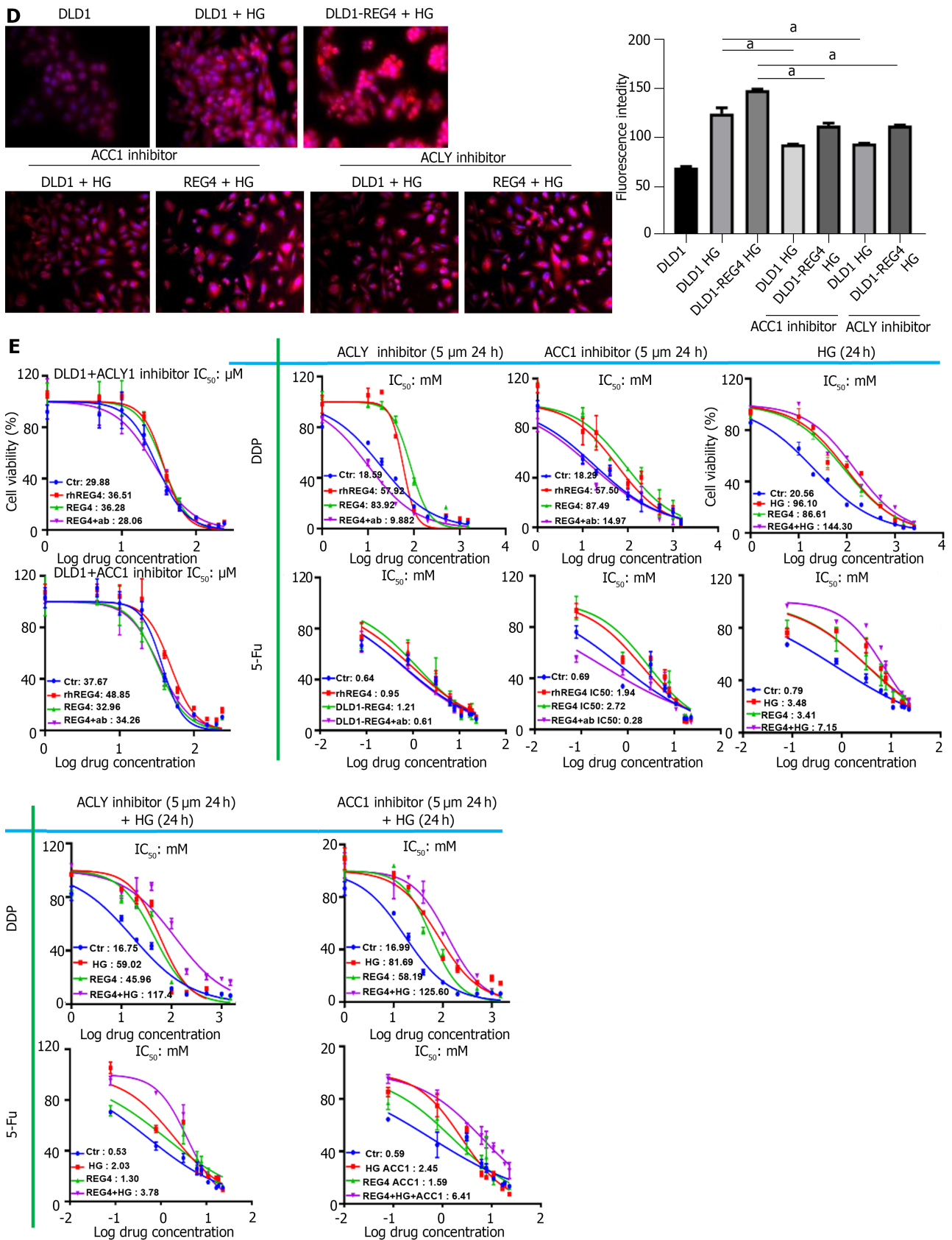


Figure 2 Effects of regenerating gene 4 on chemoresistance and droplet formation of DLD-1 cells. A: After treatment with cisplatin (DDP) or 5-fluorouracil (5-FU), the viability was measured in DLD-1 cells, DLD-1 cells treated with recombinant human regenerating gene 4 (REG4) (rhREG4), DLD-1 cells transfected with full-length (FL)-REG4 plasmid, and DLD-1 cells treated with anti-REG4 antibody; B: The lipid droplet level was measured in DLD-1 cells, DLD-1 cells treated with rhREG4, DLD-1 cells transfected with FL-REG4 plasmid, and DLD-1 cells treated with anti-REG4 antibody; C: REG4 expression was detected in DLD-1, chemoresistant DLD-1 cells to DDP and 5-FU by western blot, proteins related to de novo synthesis or assembly pathway of lipid droplets were also detected in DLD-

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. ^a*P* < 0.001; ^b*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.

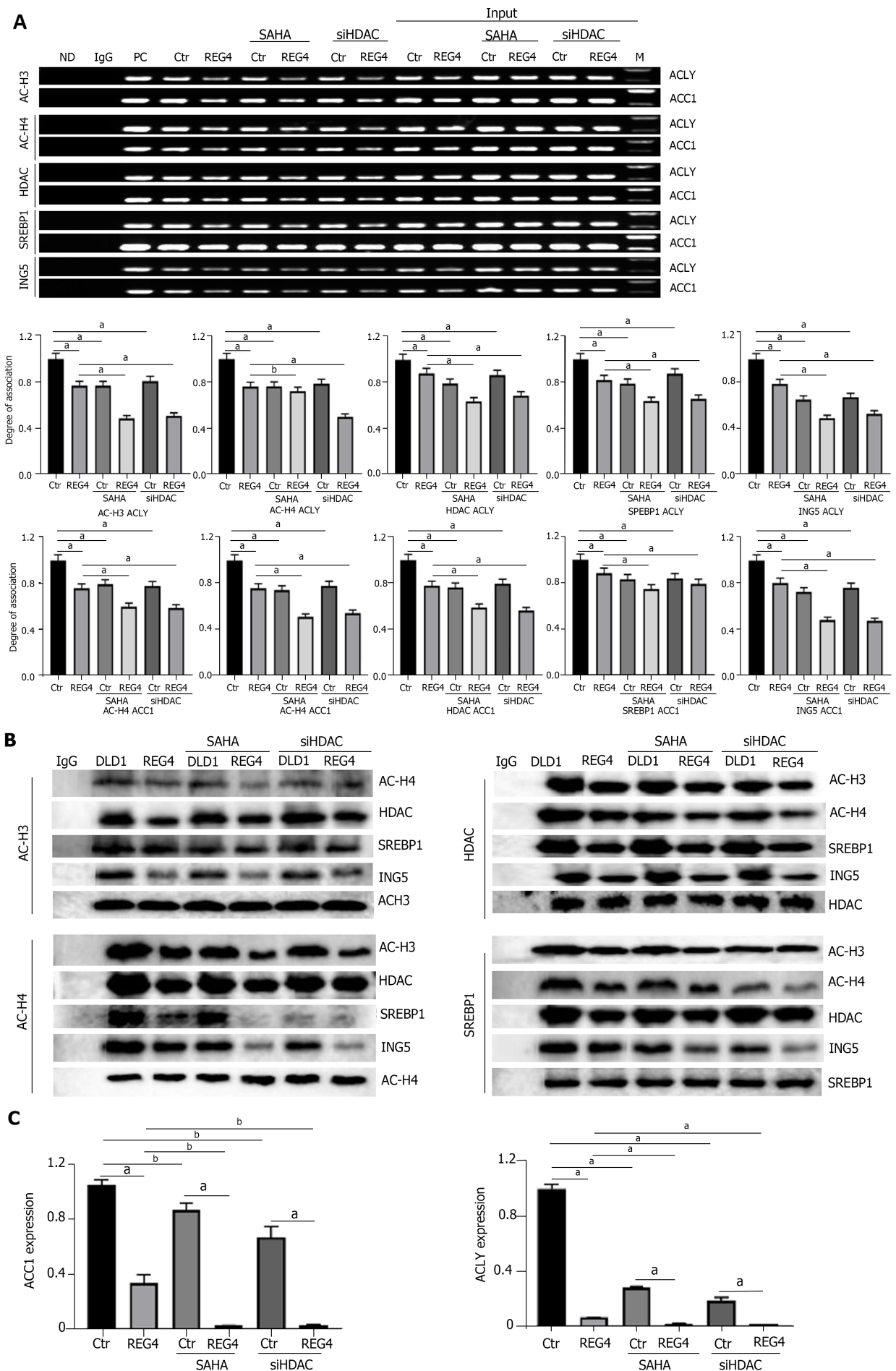
antibody dramatically reduced the autocrine and paracrine effects of secretory *REG4* on the ability of colon cancer cells to invade, migrate and proliferate[19]. *REG4* overexpression may cause resistance to the irradiation-induced apoptosis of colon cancer cells. Animal studies have shown that rh*REG4* 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].

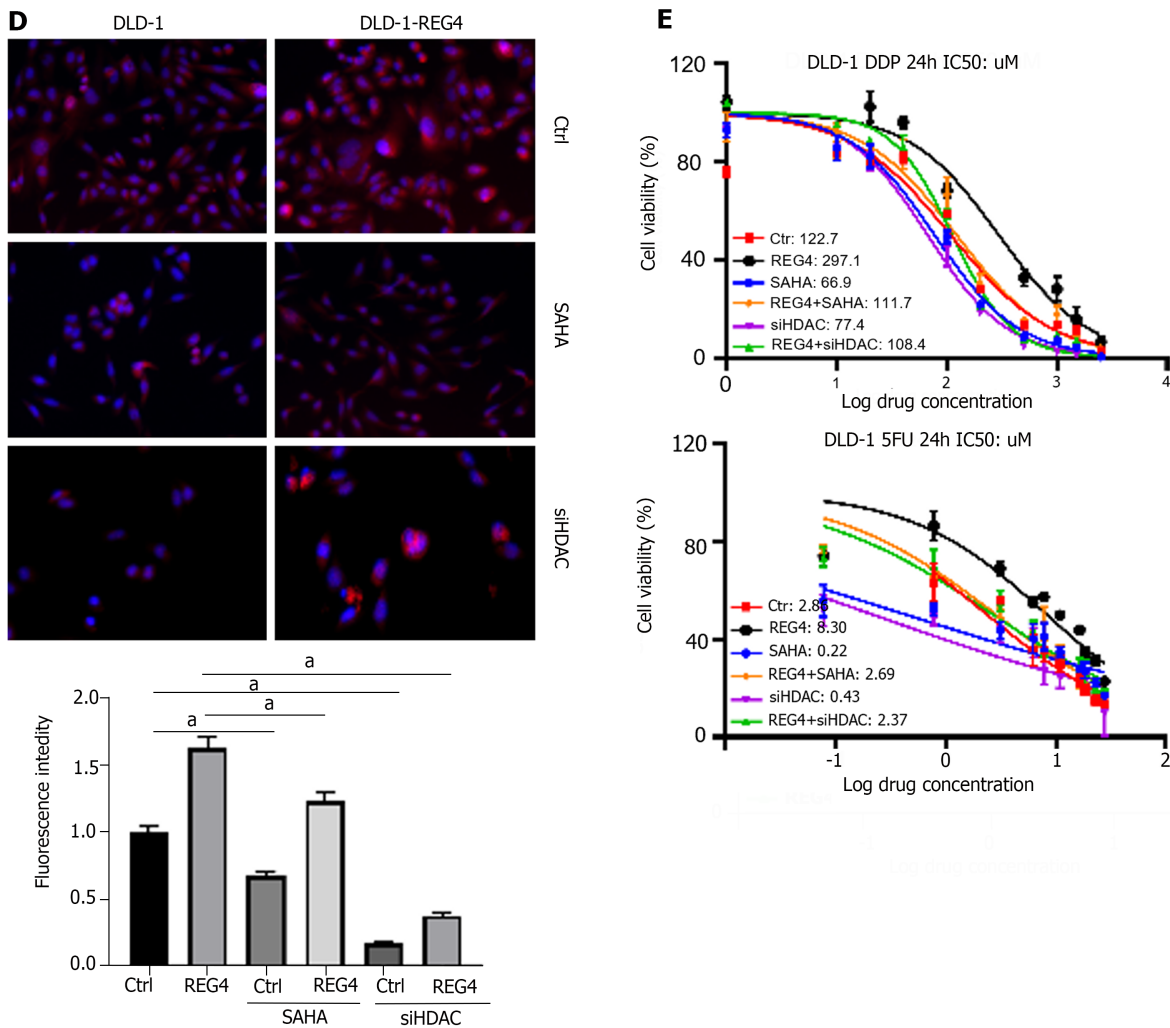
We conducted bioinformatics analysis, meta-analysis, and pathological and serological studies to evaluate the clinicopathological and prognostic significance of *REG4* expression in CRC. In bioinformatics analysis, we used data of different sizes and from different sources to ensure data heterogeneity, 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^{bright} 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 γ -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/ β -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- κ B, p-NF- κ 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- κ 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



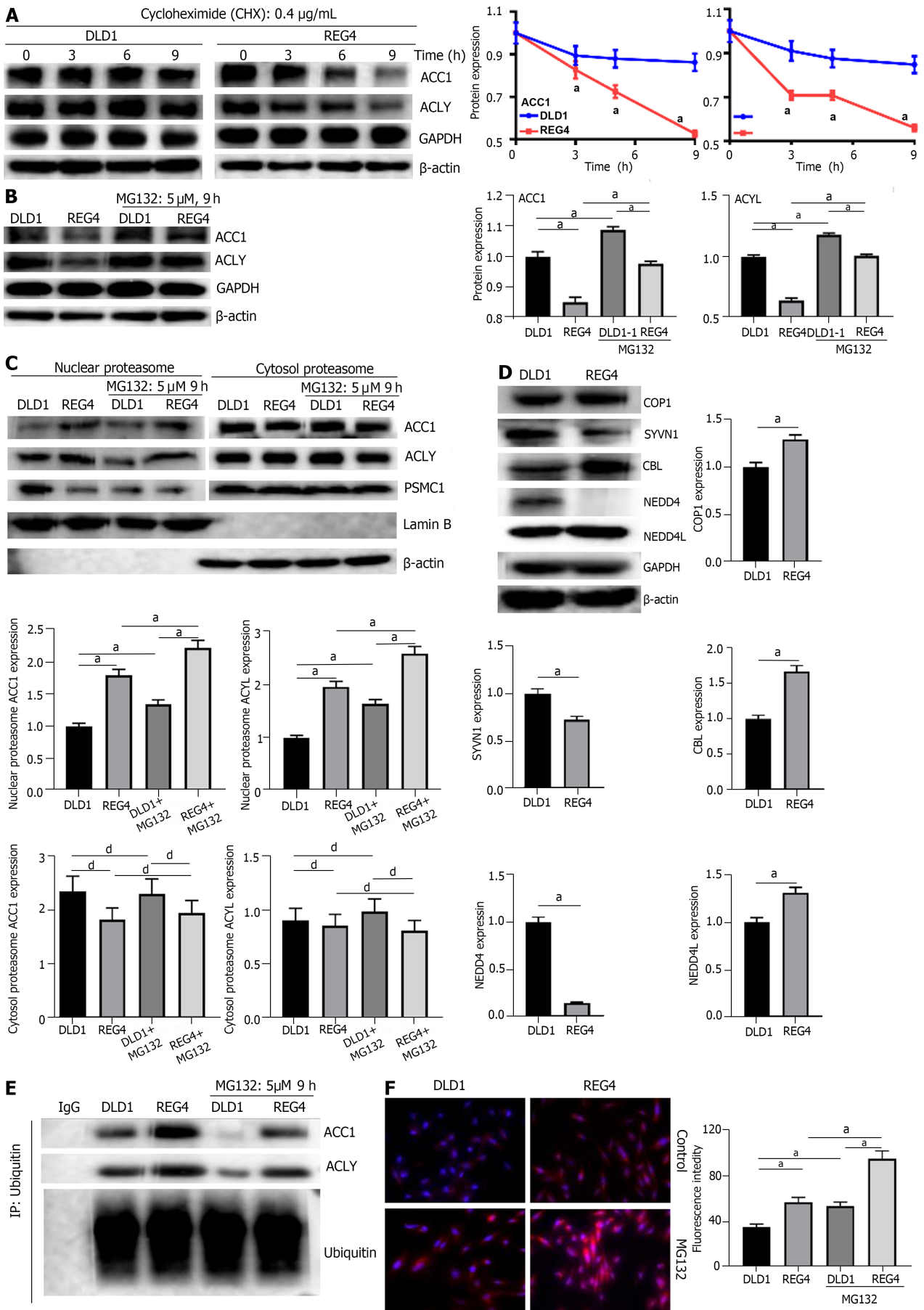


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Figure 3 Full-length regenerating gene 4 weakened the transcription of acetyl-CoA carboxylase 1 and ATP-citrate lyase. A: DLD-1 cells and full-length-regenerating gene 4 (FL-REG4) transfectants were treated with suberoylanilide hydroxamic acid (SAHA) 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. ^a*P* < 0.001; ^b*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 α [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 IC₅₀ 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.



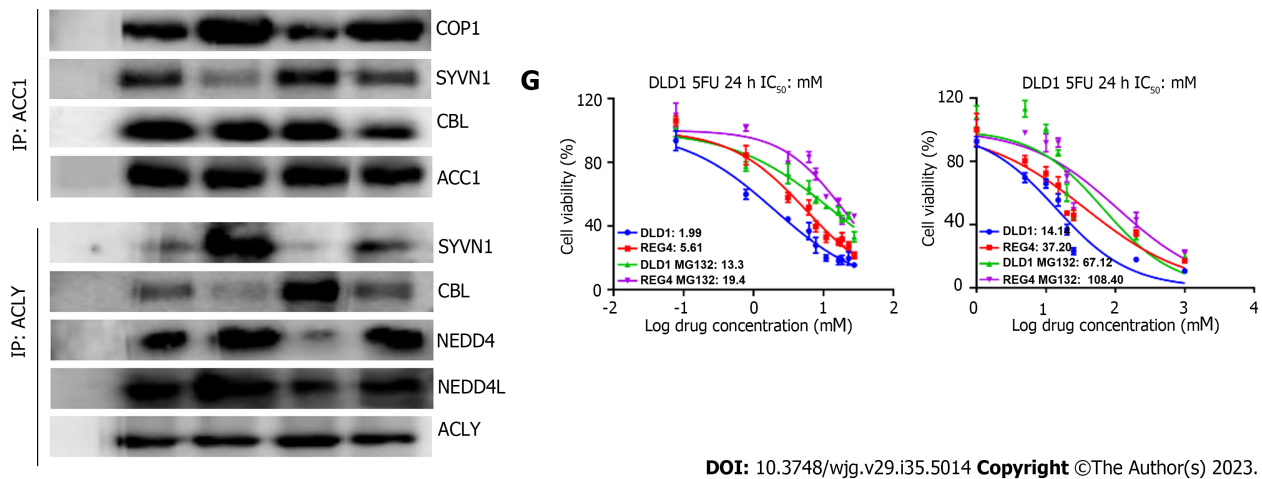


Figure 4 Full-length regenerating gene 4 destabilized acetyl-CoA carboxylase 1 and ATP-citrate lyase proteins via proteasomal degradation.

A: DLD-1 cells and full-length-regenerating gene 4 (FL-*REG4*) transfectants were treated with cycloheximide (0.4 $\mu\text{g/mL}$), followed by western blotting to detect acetyl-CoA carboxylase 1 (ACC1) and ATP-citrate lyase (ACYL) expression; B: DLD-1 cells and FL-*REG4* transfectants were treated with MG132 (5 μM , 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. $^{\ast}P < 0.001$; $^{\circ}$ No significance. *REG4*: Regenerating gene 4; ACC1: Acetyl-CoA carboxylase 1; ACCY: 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, G₂/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- κ 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- κ 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

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