89837-Answering Reviewers

Dear editors and reviewers:

Thank you very much for giving us an opportunity to revise our manuscript. We appreciate the editors and reviewers very much for their constructive comments and suggestions on our manuscript entitled "Bile acids- induced farnesoid X receptor - BTB and CNC homology1- glutathione axis upregulation inhibits ferroptosis sensitivity in gastric cancer cells" (Manuscript NO: 89837).

We have studied reviewers' comments carefully. According to the reviewers' detailed suggestions, we have made a careful revision on the original manuscript. All revised contents are highlighted with yellow in the revised manuscript which we would like to submit for your kind consideration. The main corrections are in the manuscript and the responds to the reviewers' comments are as follows (the replies are highlighted in blue).

Replies to the reviewers' comments:

Reviewer #1:
Scientific Quality: Grade B (Very good)
Language Quality: Grade B (Minor language polishing)
Conclusion: Minor revision
Specific Comments to Authors: During gastric cancer development in both humans and animals, bile acids serve as signaling molecules that induce metabolic reprogramming. This confers additional cancer phenotypes, including ferroptosis sensitivity. Ferroptosis is a novel mode of cell death characterized by lipid peroxidation that contributes universally to malignant progression. However, it is not fully defined if bile acids can influence gastric cancer progression by modulating ferroptosis. In this study, the authors

treated gastric cancer cells with various stimuli and evaluated the effect of bile acids on the sensitivity to ferroptosis, and aimed to reveal the mechanism of bile acids regulation in ferroptosis of gastric cancer cells. This study is well designed and performed. The results are very interesting. The reviewer recommends to accept this study after a minor revision. Comments: 1. The manuscript requires a minor editing. 2. Some Greek characters seems can't be read, please take attention about it. 3. Quality of images should be improved.

1. The manuscript requires a minor editing.

Response: We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. And here we did not list the changes but highlighted with yellow in the revised paper. We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval.

2. Some Greek characters seems can't be read, please take attention about it.

Response: We have corrected them in this revised manuscript and highlighted with yellow.

3. Quality of images should be improved.

Response: We have resubmitted all the images and figures with higher resolution and quality in an editable format.

Science editor:

The manuscript has been peer-reviewed, and it' s ready for the first decision.

Company editor-in-chief:

I have reviewed the Peer-Review Report, all of which have met the basic publishing requirements of the World Journal of Gastroenterology, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial

Office's comments and the Criteria for Manuscript Revision by Authors. Before final acceptance, uniform presentation should be used for figures showing the same or similar contents; for example, "Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...". Please provide decomposable Figures (in which all components are movable and editable), organize them into a single PowerPoint file. Please check and confirm whether the figures are original (i.e. generated de novo by the author(s) for this paper). If the picture is 'original', the author needs to add the following copyright information to the bottom right-hand side of the picture in PowerPoint (PPT): Copyright ©The Author(s) 2023. When revising the manuscript, it is recommended that the author supplement and improve the highlights of the latest cutting-edge research results, thereby further improving the content of the manuscript. To this end, authors are advised to apply PubMed, or a new tool, the RCA, of which data source is PubMed. RCA is a unique artificial intelligence system for citation index evaluation of medical science and life science literature. In it, upon obtaining search results from the keywords entered by the author, "Impact Index Per Article" under "Ranked by" should be selected to find the latest highlight articles, which can then be used to further improve an article under preparation/peer-review/revision. Please visit our RCA database for more information at: https://www.referencecitationanalysis.com/, or visit PubMed at: https://pubmed.ncbi.nlm.nih.gov/. Before its final acceptance, please provide and upload the following important documents: Biostatistics Review Certificate, a statement affirming that the statistical review of the study was performed by a biomedical statistician; Institutional Review Board Approval Form or Document, the primary version (PDF) of the Institutional Review Board's official approval, prepared in the official language of the authors' country; Institutional Animal Care and Use Committee Approval Form or Document, the primary version (PDF) of the Institutional Animal Care and Use Committee's official approval in the official language of the authors' country; The ARRIVE Guidelines, an important document related to

manuscript writing of basic research using animals. However, the quality of the English language of the manuscript does not meet the requirements of the journal. Before final acceptance, it is recommended that the author(s) provide the English Language Certificate issued by a professional English language editing company. Please visit the following website for the professional English language editing companies we recommend: https://www.wjgnet.com/bpg/gerinfo/240.

Response: We have standardized the format of figures and provided the decomposable Figures with copyright infomation in PPT. In the manuscript, we cited and discussed recent relevant research hotspots, which were cited from PubMed. We have uploaded the Biostatistics Review Certificate. Because in this study we performed only cellular experiments and did not performed clinical and animal experiments, we did not provide the Institutional Review Board Approval Form or Document, the Institutional Animal Care and Use Committee Approval Form or Document or the The ARRIVE Guidelines. As for the quality of English language of this manuscript, we had a native-English speaker that edited the manuscript for grammar, sentence structure, word usage, spelling, capitalization, punctuation, format, and general readability to improve the manuscript. And made some changes in the manuscript, these changes will not influence the content and framework of the paper. And here we did not list the changes but highlighted with yellow in revised paper. We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval.

Once again, thank you very much for your constructive comments and suggestions which would help us both in depth to improve the quality of the paper.

Kind regards,

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Basic Study

Bile acids- induced farnesoid X receptor - BTB and CNC homology1-glutathione axis upregulation inhibits ferroptosis sensitivity in gastric cancer cells

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Author contributions: Mingwei Zhong, Jiankang Zhu and Guangyong Zhang designed and coordinated the study; Chuxuan Liu, Ying Gao, Xin jin and Yun Zhang performed the experiments, acquired and analyzed data; Chuxuan Liu, Qian Xu and Huanxin Ding,interpreted the data; Chuxuan Liu, Bingjun Li, Fangke Du, Linchuan Li and Guangyong Zhang wrote the manuscript; Xiufang Xu revised this study; all authors approved the final version of the article.

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Abstract

BACKGROUND

Gastric cancer (GC) is associated with high mortality rates. Bile acids (BAs) reflux is a well-known risk factor for GC, but the specific mechanism remains unclear. During GC development in both humans and animals, BAs serve as signaling molecules that induce metabolic reprogramming. This confers additional cancer phenotypes, including ferroptosis sensitivity. Ferroptosis is a novel mode of cell death characterized by lipid peroxidation that contributes universally to malignant progression. However, it is not fully defined if BAs can influence GC progression by modulating ferroptosis.

AIM

To reveal the mechanism of BAs regulation in ferroptosis of gastric cancer cells.

METHODS

In this study, we treated GC cells with various stimuli and evaluated the effect of BAs on the sensitivity to ferroptosis. We used gain and loss of function assays to examine the impacts of farnesoid X receptor (FXR) and BTB and CNC homology1 (BACH1) overexpression and knockdown to obtain further insights into the molecular mechanism involved.

RESULTS

Our data suggested that BAs could reverse erastin-induced ferroptosis in GC cells. This effect correlated with increased glutathione (GSH) concentrations, a reduced glutathione to oxidized glutathione (GSH/GSSG) ratio, and higher glutathione peroxidase 4 (GPX4) expression levels. Subsequently, we confirmed that BAs exerted these effects by activating FXR, which markedly increased the expression of GSH synthetase and GPX4. Notably, BACH1 was detected as an essential intermediate molecule in the promotion of GSH synthesis by BAs and FXR. Finally, our results suggested that FXR could significantly promote

GC cell proliferation, which may be closely related to its anti-ferroptosis effect.

CONCLUSION

This study revealed for the first time that BAs could inhibit ferroptosis sensitivity through the FXR-BACH1-GSH-GPX4 axis in GC cells. This work provided new insights into the mechanism associated with BA-mediated promotion of GC and may help identify potential therapeutic targets for GC patients with BAs reflux.

Key words: gastric cancer; ferroptosis; bile acids; CDCA; FXR; glutathione.

Core tip: Gastric cancer (GC) is the fifth most common cancer worldwide and the third leading cause of cancer-related deaths.Bile acids (BAs) reflux is an essential carcinogenic factor in GC, but its role has not been absolutely elaborated. BAs could serve as signaling molecules to regulate the metabolic state in cells, which is closely related to ferroptosis. In the present experiment, we explored the role of BAs in the regulation of ferroptosis in GC cells. Our data suggested that BAs could significantly inhibit the ferroptosis sensitivity of GC cells and that this effect was exerted through the activation of the FXR-BACH1-GSH-GPX4 axis.

INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer worldwide and the third leading cause of cancer-related deaths because of the difficulties associated with early diagnosis [1]. Along with the improvement of life conditions, there is a noticeable decrease in the prevalence of Helicobacter pylori (H. pylori) infection, which is the major causative factor of GC [2]. Bile acids (BAs) reflux, another etiologic factor for developing GC, is receiving more attentions[3]. BAs are cholesterol-derived sterols and are essential small molecule metabolites in the human body. They are amphiphilic and can thus participate in the absorption and secretion of cholesterol in the intestines [4]. Previous work has shown that BAs reflux is an independent risk factor for precancerous gastric lesions and gastric carcinogenesis [5, 6]. For example, gastric mucosal damage can be induced by BAs through activation of the IL-6/JAK1/STAT3 pathway [5]. However, the mechanism by which BAs can promote GC progression remains unknown.

By activating BAs receptors, BAs can modulate immune responses, gastrointestinal mucosal

barrier function, gestation, metabolic diseases, and carcinogenesis [7-10]. There are many types of BAs receptors, among which the farnesoid X receptor (FXR, NR1H4) is typical and has been well investigated. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the two predominant BAs in the human body [11], the latter of which is the most potent physiologic agonist of FXR [12]. FXR activation can remodel the metabolic state of cells, including glucose metabolism and lipid metabolism, which in turn is involved in the development of a variety of metabolic diseases and cancers, such as hepatocellular carcinoma [13]. However, further research on the role of FXR in GC patients with BAs reflux is required.

The altered metabolic state, also known as metabolic reprogramming, is a vital factor in cancer progression [14]. Ferroptosis, a process that is closely related to metabolism, may be involved in the effects of BAs and FXR in GC [15]. Ferroptosis is a novel type of cell death characterized by intracellular phospholipid peroxidation, distinct from apoptosis, pyroptosis, necroptosis, and autophagy[16, 17]. This unique mode of cell death is regulated by a variety of factors, among which oxidative stress homeostasis is particularly influential. Glutathione peroxidase 4 (GPX4) specifically recognizes peroxidized lipids and scavenges them by converting reduced glutathione (GSH) to oxidized glutathione (GSSG) for anti-ferroptosis [18, 19]. Therefore, GSH, as the substrate of GPX4, also has a key role in the resistance to ferroptosis. Changes in GSH metabolism will eventually lead to alterations in cellular sensitivity to ferroptosis [20]. Although ferroptosis has been reported in GC development and treatment [21, 22], few studies have described ferroptosis in GC with BAs reflux.

In the present study, we investigated the role of BAs, especially CDCA, in the regulation of ferroptosis sensitivity in GC. We subsequently identified the specific receptors for these BAs and further investigated the molecular mechanism.

MATERIALS AND METHODS

Reagents and antibodies

Cholic acid (CA, S3742), dehydrocholic acid (DCA, S4562), chenodeoxycholic acid (CDCA, S1843), erastin (S7242), Ferrostatin-1 (Fer-1, S7243), and GW4064 (S2782) were purchased from Selleck Chemicals (Houston, TX, USA). RSL3 (HY-100218A) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Anti-GPX4 (67763-1-Ig, 1:2500), anti-β-actin (HRP-66009, 1:5000), anti-FXR (25055-1-Ig, 1:1000), anti-GCLC (12601-1-AP, 1:4000), anti-GCLM (14241-1-AP, 1:4000), anti-GSS (67598-1-Ig, 1:4000), and anti-BACH1 (14018-1-AP, 1:5000) antibodies were purchased from Proteintech (Wuhan, China).

Cell culture

HGC-27 and MKN-45 cells were purchased from Procell (Wuhan, China) and cultured in

MEM (for HGC-27) and RPMI-1640 (for MKN-45) medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% Penicillin/Streptomycin (Gibco) at 37°C and 5% CO2. The cell lines were correctly identified by short tandem repeat (STR) analysis and periodically tested for mycoplasma.

Cell transfection

Cells were seeded in 6-well plates (5×10^5 cells/well) and incubated for 18 hours. Then, overexpression or short hairpin RNA (shRNA) plasmids for the indicated genes were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 48 hours according to the manufacturer's instructions.

Cell viability assay

Cells were seeded in 96-well plates (5,000 cells/well) in complete medium. After incubation for 18 hours, the indicated treatments were added to the cells and incubated for certain times. Then, 100 μL complete medium containing 10 μL cell count kit-8 (CCK-8) reagent (CK04, Dojindo Laboratories, Kumamoto, Japan) was added to each well. After incubating the cells for 2 hours, the absorbance value for each well was colorimetrically measured at a wavelength of 450 nm.

GSH and Malondialdehyde (MDA) assay quantification

The cells were collected after indicated stimuli. The GSH concentrations and GSH/GSSG ratio were quantified using the GSSG/GSH Quantification Kit (G263, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The results were quantified colorimetrically at a wavelength of 405 nm.

After the indicated treatments, the cells were collected and assayed using the MDA Assay Kit (S0131S, Beyotime, Shanghai, China) following the manufacturer's instructions to measure the levels of MDA. The results were quantified colorimetrically at a wavelength of 532 nm.

Lipid reactive oxygen species (ROS) assay

After the indicated treatments, BODIPY-589/591 C11 (D3861, Thermo Fisher Scientific, Waltham, MA, USA) was added to each well (10 μM). After incubated at 37°C for 30 minutes, the cells were washed with PBS for three times. Subsequently, the nuclei were stained with DAPI (C1002, Beyotime) for 30 minutes at room temperature. Finally, the lipid ROS was

observed under a fluorescence microscope with 488 nm excitation.

5-ethynyl-2'-deoxyuridine (EdU) Staining

Cell proliferation rates under different treatment conditions were assessed using EdU assays (Beyotime) according to the manufacturer's instructions.

Colony formation assay

Cells were seeded in 6-well plates (500 cells/well), treated with various stimuli, and incubated for 10 to 14 days. The cells were then rinsed three times with PBS and fixed with 4% paraformaldehyde at room temperature for 30 minutes. Subsequently, the fixed cells were treated with crystal violet at 4°C overnight.

Western blot (WB)

Cells were lysed using RIPA buffer containing 1% Phenylmethanesulfonyl fluoride (PMSF, ST505, Beyotime, Shanghai, China) and 2% phosphatase inhibitor. The total protein concentration was quantified using the Bicinchoninic Acid (BCA) Protein Assay Kit (ST505, Thermo Fisher Scientific, Waltham, MA, USA). Next, protein samples (30 μg) were separated using 10% SDS-PAGE (PG212, EpiZyme, Shanghai, China). Then, the proteins were transferred to PVDF membranes, followed by blocking with 5% BSA (A8020, Solarbio, Beijing, China) at room temperature for 1 hour. Afterwards, the membranes were incubated with primary antibodies at 4°C overnight. Subsequently, the membranes were washed with PBST and incubated with a goat anti-mouse or goat anti-rabbit secondary antibody for 1 hour at room temperature. Finally, the protein bands were visualized with ECL (Millipore) and quantified with ImageJ software (National Institutes of Health) the manufacturer's instructions.

Statistical analyses

SPSS 22.0 software (Chicago, IL, USA) was used for data analysis. GraphPad Prism 8.0 (San Diego, CA, USA) software was used to create the images. Data are presented as mean ± standard deviation. One-way ANOVA was used to compare the differences between groups. A P-value of less than 0.05 indicated statistical significance.

RESULTS

BAs can promote GC cell proliferation and inhibit erastin-induced ferroptosis sensitivity in GC cells

It has been shown that BAs tend to induce gastric intestinal metaplasia prior to causing GC [23]. Thus, two GC cell lines, HGC-27 and MKN-45, were chosen because they were both classified as intestinal type GC cells [24]. Three common BAs including CA, DCA, and CDCA, were chosen to stimulate GC cells *in vitro*. The cell viability assay results suggested that these BAs could significantly promote GC cell proliferation rates, especilly CDCA (Figure 1A). Subsequently, to investigate if they could modulate ferroptosis in GC cells, we examined the effects of the three BAs on HGC-27 and MKN-45 cell sensitivity to erastin, a classical inducer of ferroptosis. Interestingly, the GC cells treated with BAs exhibited higher viabilities compared with the controls, suggesting that the BAs possibly could support resistance to the ferroptosis induced by erastin (Figure 1B). Because it was the most effective BAs proved by above results and in previous study [23], CDCA was chosen in subsequent experiments. We then examined the effect of CDCA on the sensitivity to erastin-induced ferroptosis in GC cells at 24 and 48 hours, respectively. The anti-ferroptosis effect was confirmed (Figure 1C and D). To exclude interference from other types of cell death, we performed the MDA assays (Figure 1E) and BODIPY-589/591 C11 staining (Figure 1F), which directly reflected ferroptosis and reconfirmed the anti-ferroptosis function of the BAs.

BAs significantly upregulated GSH and GPX4 levels in GC cells

The cystine-glumate antiporter(xCT) is an essential anti-ferroptosis protein located on the cytomembrane that exchanges intracellular glutamate for extracellular cystine in a 1:1 ratio [25, 26]. Mechanistically, erastin induces ferroptosis by acting on xCT and inhibiting its function. This thereby downregulates the levels of downstream GSH and GPX4, which inhibit the onset of ferroptosis [27]. Additionally, another classical ferroptosis inducer is RSL3, which targets and inactivates GPX4 [28]. Therefore, to explore the anti-ferroptosis mechanism of CDCA, we examined its effect on RSL3-induced cell death using cell viability assays. Interestingly, CDCA did not ameliorate RSL3-induced GC cell death (Figure 2A), nor could it

ameliorate the ferroptosis caused by RSL3 (Figure 2B and C). We therefore speculated that CDCA possibly exerted its anti-ferroptosis effect by upregulating GSH and GPX4 levels. To verify this hypothesis, we examined the GSH concentrations and GSH/GSSG ratio in CDCA-treated cells, finding that CDCA treatment significantly increased them compared with the control group (Figure 2D and E). Besides, CDCA also significantly attenuated the GPX4 protein expression downregulation induced by erastin, as seen with WB analysis (Figure 2F).

BAs exerted its anti-ferroptosis sensitivity function in GC cells by activating FXR

CDCA is the strongest FXR agonist in the human body [12]. Therefore, we hypothesized that CDCA acted through activiting FXR to inhibit the sensitization of GC cells to ferroptosis. We firstly used GW4064, an *in vitro* agonist of FXR, and found that the ferroptosis sensitivity of GC cells treated with GW4064 was significantly reduced (Figure 3A). Subsequently, we transfected shFXR and its control plasmid in HGC-27 and MKN-45 cells, constructing a cellular knockdown model of FXR to be successfully constructed by WB analysis (Figure 3B). Our data showed that after knocking down FXR, CDCA-induced erastin resistance was not observed (Figure 3C) and it could no longer reverse the onset of erastin-induced ferroptosis (Figure 3D and E). We further constructed an overexpression model of FXR in HGC-27 and MKN-45 cells (Figure 3F). FXR overexpression resulted in a significant enhancement of resistance to erastin-induced cell death in HGC-27 and MKN-27 (Figure 3G), as well as a significant reversal of ferroptosis (Figure 3H and I).

FXR significantly promoted GSH synthesis in GC cells

To investigate whether FXR could likewise increase intracellular GSH concentrations, we examined the effect of FXR on GSH levels. The results showed that GSH concentrations were significantly reduced after FXR knockdown in HGC-27 and MKN-45 cells (Figure 4A). The GSH/GSSG ratio, an indicator of cellular antioxidant capacity, was also significantly decreased after FXR knockdown (Figure 4B). We next examined the effect of FXR on the

protein expression levels of GSH synthesis-related enzymes in GC cells using WB analysis, finding that FXR knockdown significantly reduced the expression of GSH synthases, including GCLC, GCLM and GSS. It also affected GPX4 expression levels, which used GSH as a substrate (Figure 4C). To further validate these observations, we repeated the above experiments using the FXR overexpression HGC-27 and MKN-45 cells. The results showed that overexpressing FXR in these GC cells led to increased GSH concentrations (Figure 4D), GSH/GSSG ratio (Figure 4E), and GSH synthase and GPX4 expression levels (Figure 4F).

FXR exerted its anti-ferroptosis and pro-GSH synthesis effects correlating with inhibiting BACH1 in GC cells

Recently, FXR was shown to inhibit heme catabolism and increase heme levels by repressing HO-1 transcription [29]. Heme in high concentrations can inhibit BTB and CNC homology1 (BACH1), which can lead to decreased expression of GSH synthases [30, 31]. Therefore, BACH1 is potentially a crucial bridge through which FXR exerted its effects. We firstly detected BACH1 protein expression using WB analysis in HCG-27 and MKN-45 cells with overexpression or knockdown of FXR expression. The results showed that knocking down FXR indeed significantly elevated BACH1 protein levels (Figure 5A), while overexpressing FXR significantly downregulated BACH1 expression (Figure 5B). To further validate the role of BACH1 in this system, we constructed overexpression models of BACH1 in HGC-27 and MKN-45 cells and verified (Figure 5C). We then transfected cells with the FXR overexpression plasmid together with the BACH1 overexpression plasmid and erastin treatment. This rescue experiment suggested that overexpression of BACH1 led to a significant reduction in ferroptosis resistance mediated by FXR, as seen with the MDA assay and BODIPY-589/591 C11 staining results (Figure 5D and E). Simultaneously, FXR-mediated enhancements of glutathione concentrations (Figure 5F), GSH/GSSG ratio (Figure 5G), glutathione synthase expression including GCLC, GCLM, GSS, and GPX4 (Figure 5H) were significantly reversed by overexpressed BACH1.

FXR significantly promoted GC cells proliferation

To further determine the role of FXR in GC progression, we analyzed its biological functions in GC cells. As described above, knockdown models of FXR in HGC-27 and MKN-45 cells were constructed (Figure 3B). Subsequently, cell viability assays showed that GC cell proliferation rates were significantly reduced after FXR knockdown (Figure 6A). This was also confirmed by EdU staining, which showed that the proportion of actively proliferating GC cells was significantly reduced with lower FXR expression levels (Figure 6B). Additional assays likewise revealed that the colony formation ability of GC cells was significantly decreased after knocking down FXR (Figure 6C). Experiments with the overexpression model (Figure 3F) showed that FXR promoted GC cell proliferation (Figure 6D), facilitated the capacity of DNA replication (Figure 6E), and enhanced the colony formation ability (Figure 6F).

DISCUSSION

GC is a major cause of cancer-related mortality in East Asia [32], but the molecular mechanisms and regulatory systems involved still need to be further elucidated. In the present study, we provided evidence that BAs can promote GC progression by inhibiting the ferroptosis sensitivity of GC, then explored the related mechanism in more detail.

BAs are essential small molecule metabolites in humans that can act as signaling molecules in the onset and progression of many diseases, including various cancers [33]. For example, BAs can promote gastric carcinogenesis via the IL-6/JAK1/STAT3 axis [5]. Since the discovery of ferroptosis, numerous studies have focused on its therapeutic promise in cancer, including in GC [34]. For instance, activation of the Wnt/beta-catenin signaling pathway significantly enhanced ferroptosis resistance in GC [35]. ACTL6A could inhibit the onset of ferroptosis in GC by upregulating GCLC [36]. However, there are still few studies on if BAs can affect GC through regulation of ferroptosis. Our data indicated that several BAs could significantly inhibit erastin-induced cell death in GC cells, especially CDCA. Additionally, we further confirmed that the form of cell death involved was ferroptosis. Subsequently, we found that the BAs did not reverse cell death induced by RSL3, a ferroptosis inducer that targeted GPX4. This suggested to us that the site of action where BAs exerted its

anti-ferroptosis function is possibly GSH, which is between xCT, the target of erastin, and GPX4. Indeed, both the GSH concentrations and GSH/GSSG ratio were significantly elevated following BA treatment in GC cells. This suggested that the BAs increased levels of both GSH and GPX4 in the GC cells, resulting in ferroptosis resistance of GC.

To clarify the potential molecular mechanisms by which CDCA regulated ferroptosis in GC cells, we hypothesized that it acted through FXR. FXR is classified as part of the nuclear hormone receptor superfamily, for which BAs are physiological ligands. Of these, CDCA has the strongest in vivo affinity for FXR [11, 37]. Previous work demonstrated that FXR could promote gastric intestinal metaplasia, a precancerous lesion of GC, via the FXR/SNAI2/miR-1 axis [38]. However, the role played by FXR in GC progression, especially ferroptosis, remains unknown. We firstly experimentally used GW4064, a classical in vitro agonist of FXR, and found that it could exert similar effects as BAs. Subsequently, we performed FXR gain and loss of function assays. The anti-ferroptosis effect of the BAs almost disappeared after knocking down FXR, while overexpression of FXR without BAs induced an anti-ferroptosis effect in GC cells. In addition, our data suggested that FXR could increase the expression of GSH synthases, including GSS, GCLC, and GCLM, as well as significantly increase the GSH concentration, GSH/GSSG ratio, and GPX4 expression in GC cells. These results suggested that BAs could exert anti-ferroptosis effects by promoting GSH synthesis via activating FXR.

To clarify the mechanism of FXR in GC, we reviewed the relevant studies and found that FXR could suppress the expression of HO-1, which had the ability to degrade heme, leading to inhibition of BACH1 [29]. BACH1 belongs to the cap'n'collar (CNC) b-Zip family of proteins and coule inhibite the intracellular synthesis of GSH [30, 31, 39] . Therefore, we investigated if BACH1 acted as a bridge between GSH synthesis and FXR. Our data indicated that FXR expression levels were inversely related to those of BACH1, suggesting that FXR inhibited BACH1 expression. Subsequent functional rescue experiments revealed that overexpression of BACH1 partially counteracted the pro-GSH synthesis and anti-ferroptosis effects of FXR. Finally, we investigated the effect of FXR on GC cell growth, finding that FXR had remarkable oncogenic ability. FXR significantly increased GC cell proliferation rates, which may be closely related to the inhibition of ferroptosis of GC cells by FXR.

This study has some limitations. Restricted by the experimental conditions, we were unable

to perform in vivo experiments to validate these results. Additionally, the molecular mechanism of FXR-mediated regulation of BACH1 requires further research.

CONCLUSION

Overall, our study illustrated a new strategy of ferroptosis regulation by BAs in GC cells and provided new insights into the molecular mechanisms by which BAs promoted GC progression. Ferroptosis played an influential role in GC progression, raising the possibility that treatment targeting FXR and BACH1 might improve the outcomes of GC patients with BAs reflux.

ACKNOWLEDGEMENTS

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ABBREVIATIONS:

GC: gastric cancer; BAs: bile acids; FXR: farnesoid X receptor; BACH1: BTB and CNC homology1; GSH: glutathione; GSSG: oxidized glutathione ; GSH/GSSG: reduced glutathione to oxidized glutathione; GPX4: glutathione peroxidase 4; CA: cholic acid; DCA: dehydrocholic acid; CDCA: chenodeoxycholic acid; Fer-1: Ferrostatin-1; ROS: reactive oxygen species; MDA: malondialdehyde; WB: western blot; NC: negative cantroll.

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Footnotes

Conflict-of-interest statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations: GC: gastric cancer; BAs: bile acids; FXR: farnesoid X receptor; BACH1: BTB and CNC homology1; GSH: glutathione; GSSG: oxidized glutathione ; GSH/GSSG: reduced glutathione to oxidized glutathione; GPX4: glutathione peroxidase 4; CA: cholic acid; DCA: dehydrocholic acid; CDCA: chenodeoxycholic acid; Fer-1: Ferrostatin-1; ROS: reactive oxygen species; MDA: malondialdehyde; WB: western blot.

Data sharing statement: No additional data are available.

Figures

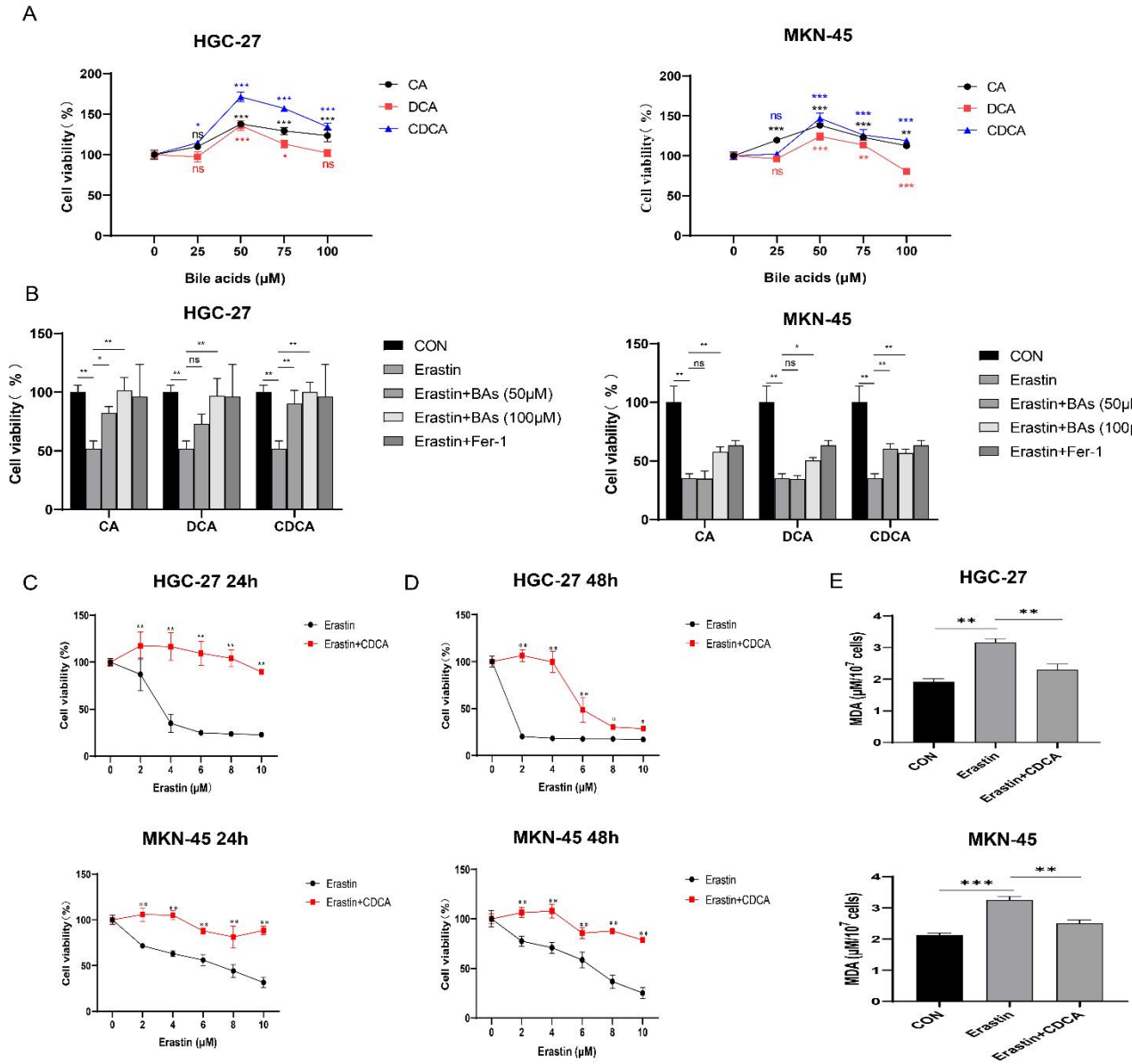
A
HGC-27
MKN-45
Cell viability (%)
Bile acids (μM)
CA
DCA
CDCA
B
HGC-27
MKN-45
Cell viability (%)
CON
Erastin
Erastin+BAs (50μM)
Erastin+BAs (100μM)
Erastin+Fer-1
C
HGC-27 24h
MKN-45 24h
D
HGC-27 48h
MKN-45 48h
Cell viability (%)
Erastin (μM)
Erastin
Erastin+CDCA
E
HGC-27
MKN-45
MDA (μM/10^7 cells)
CON
Erastin
Erastin+CDCA
F
HGC-27
MKN-45
Oxidized
Non-Oxidized
DAPI
Merge
CON
Erastin
Erastin+
CDCA

Figure 1Bile acids enhanced proliferation and inhibited erastin-induced ferroptosis sensitivity in GC cells. A: Cell viability assay for HGC-27 and MKN-45 cells treated with three BAs. B: Cell viability assay for HGC-27 and MKN-45 cells treated with different concentration of BAs together with erastin (5 μM). C, D: Cell viability assay for two GC cell lines stimulated with erastin followed by CDCA (50 μM) or control for 24 and 48 hours. E: MDA production in HGC-27 and MKN-45 cells. F: BODIPY-589/591 C11 staining to identify lipid ROS in the cell lines under different treatments. Scale bar, 100 μm. *P<0.05, **P<0.01, ***P<0.001. These experiments were repeated three times. GC: gastric cancer; BAs: bile acids; CDCA: chenodeoxycholic acid; MDA: malondialdehyde.

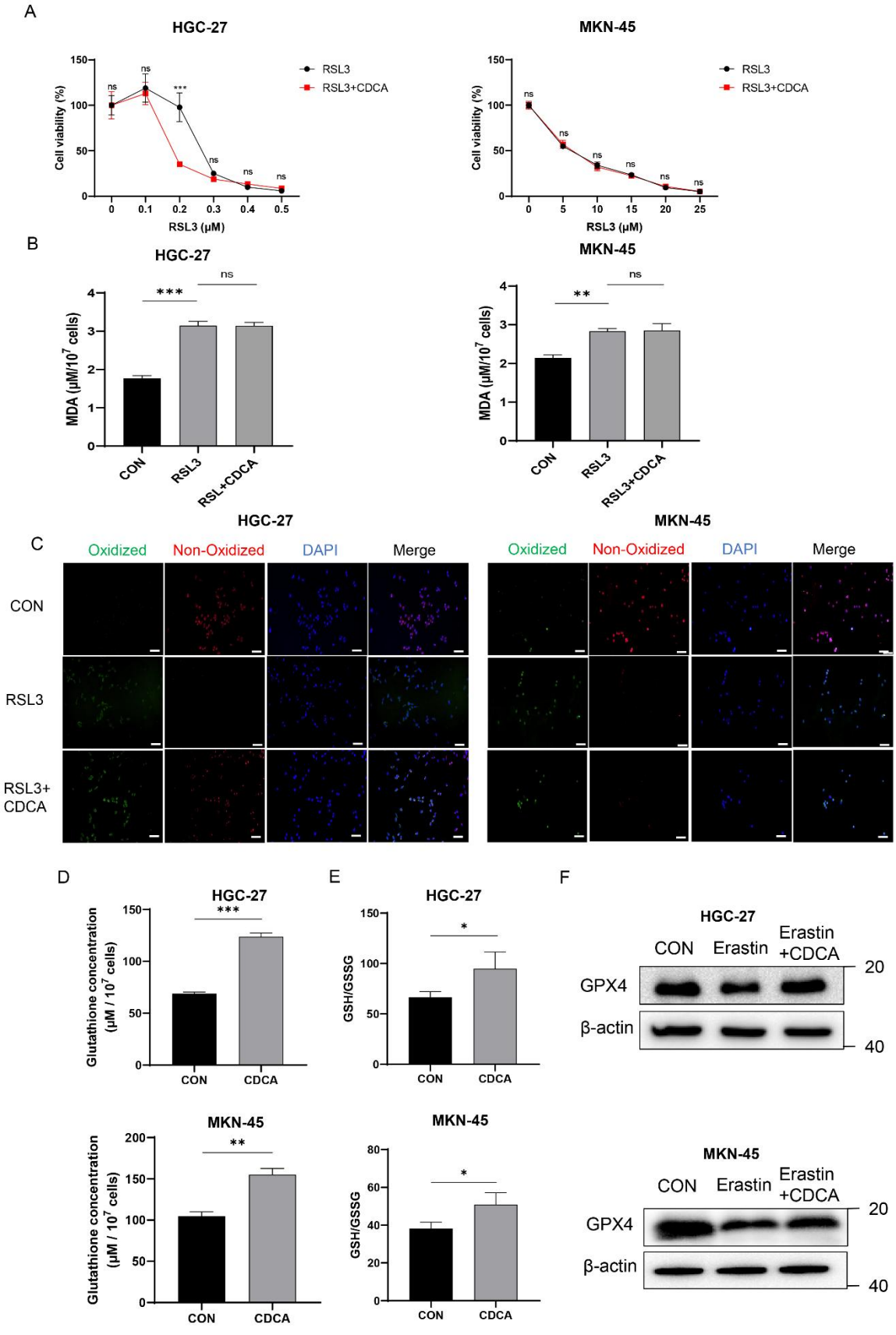
A
HGC-27
MKN-45
RSL3
RSL3+CDCA
Cell viability (%)
RSL3 (μM)
B
HGC-27
MKN-45
MDA (μM/10^7 cells)
CON
RSL3
RSL+CDCA
RSL3+CDCA
C
Oxidized
Non-Oxidized
DAPI
Merge
CON
RSL3
RSL3+ CDCA
D
HGC-27
MKN-45
Glutathione concentration (μM / 10^7 cells)
CON
CDCA
E
HGC-27
MKN-45
GSH/GSSG
F
HGC-27
MKN-45
CON
Erastin
Erastin +CDCA
GPX4
β-actin
20
40

Figure 2BAs significantly upregulated GSH and GPX4 in GC cells. A: Cell viability assay of two GC cell lines treated with RSL3 together with CDCA or control. B: MDA production in HGC-27 and MKN-45 cells treated with RSL3 (0.2 μM for HGC-27, 10 μM for MKN-45) followed by CDCA or control. C: BODIPY-589/591 C11 staining to identify lipid ROS in the cell lines treated with RSL3 (0.2 μM for HGC-27, 10 μM for MKN-45) followed by CDCA or control. D: The GSH concentrations were measured in cells treated with CDCA. E: The GSH/GSSG ratio was measured in cells treated with CDCA. F: Western blot analysis of GPX4 protein expression in HGC-27 and MKN-45 cells under different stimuli. Scale bar, 100 μm. $*P<0.05$, $**P<0.01$, $***P<0.001$. These experiments were repeated three times. GC: gastric cancer; CDCA: chenodeoxycholic acid; MDA: malondialdehyde; GSH: glutathione; GSSG: oxidized glutathione ; GSH/GSSG: reduced glutathione to oxidized glutathione; GPX4: glutathione peroxidase 4.

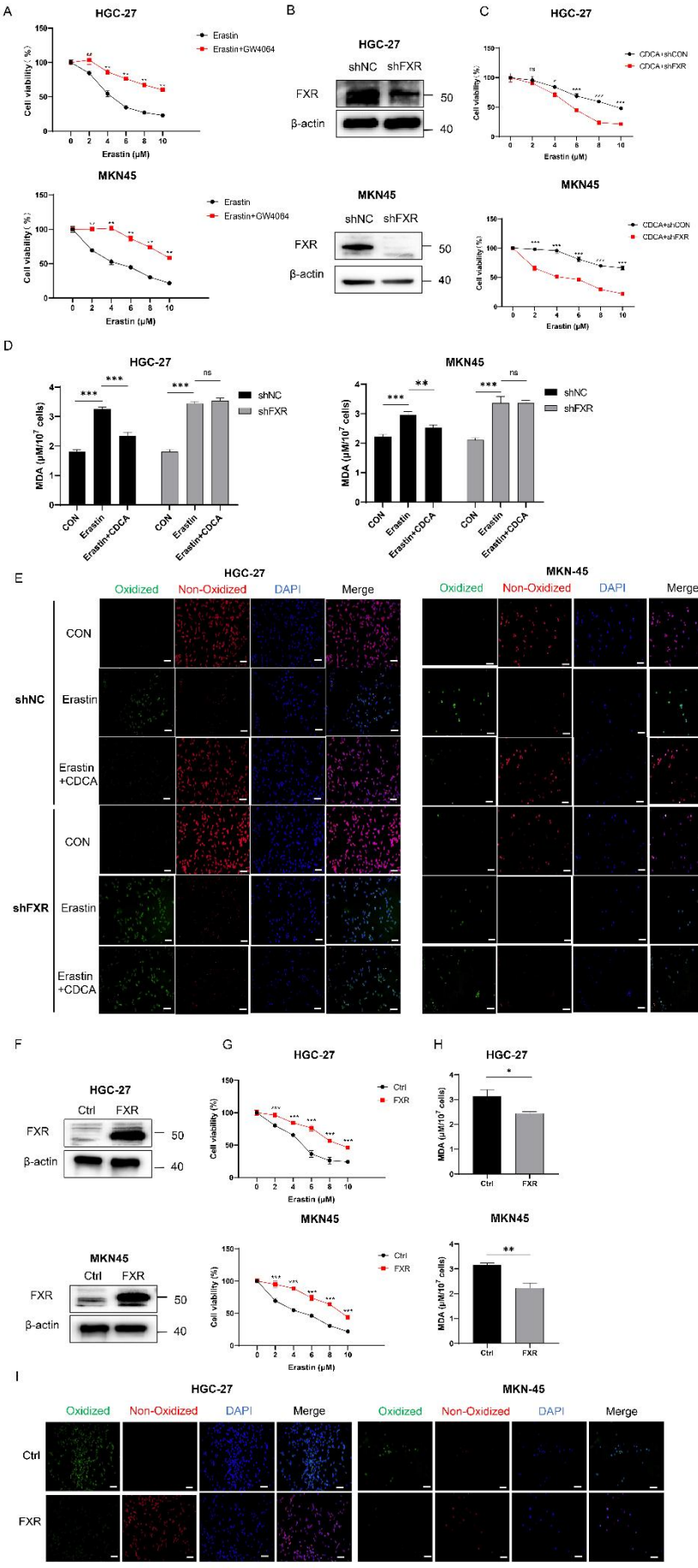
A
HGC-27
Erastin
Erastin+GW4064
Cell viability(%)
Erastin (μM)
MKN45
B
HGC-27
shNC shFXR
FXR
β-actin
50
40
MKN45
C
HGC-27
CDCA+shCON
CDCA+shFXR
MKN45
D
HGC-27
MKN45
shNC
shFXR
MDA (μM/10^7 cells)
CON
Erastin
Erastin+CDCA
E
HGC-27
MKN-45
Oxidized
Non-Oxidized
DAPI
Merge
shNC
shFXR
CON
Erastin
Erastin +CDCA
F
HGC-27
Ctrl FXR
MKN45
G
HGC-27
MKN45
Ctrl
FXR
H
HGC-27
MKN45
I
HGC-27
MKN-45
Ctrl
FXR

Figure 3BAs inhibited ferroptosis sensitivity of GC cells by activating FXR. A: Cell viability of erastin-treated HGC-27 and MKN-45 cells with or without GW4064 treatment. B: HGC-27 and MKN-45 cells were transfected with shFXR or shNC plasmid. Successful construction was confirmed by western blot analysis. C: Cell viability assay of GC cells treated with different concentrations of erastin and CDCA (50 μM) transfected with shFXR or shNC for 24 hours. D, E: MDA production and BODIPY-589/591 C11 staining of GC cells transfected with shFXR or shNC plasmid and treated with erastin together with or without CDCA for 24 hours. F: GC cells were transfected with control or FXR-coding plasmid and confirmed through western blot analysis. G: Cell viability assay of GC cells treated with different concentrations of erastin and CDCA (50 μM) transfected with control or FXR-coding plasmid for 24 hours. H, I: MDA production and BODIPY-589/591 C11 staining of GC cells transfected with control or FXR-coding plasmid and treated with erastin together with or without CDCA for 24 hours. Scale bar, 100 μm. $*P<0.05$, $**P<0.01$, $***P<0.001$. These experiments were repeated three times. GC: gastric cancer; FXR: farnesoid X receptor; NC: negative control; CDCA: chenodeoxycholic acid; MDA: malondialdehyde.

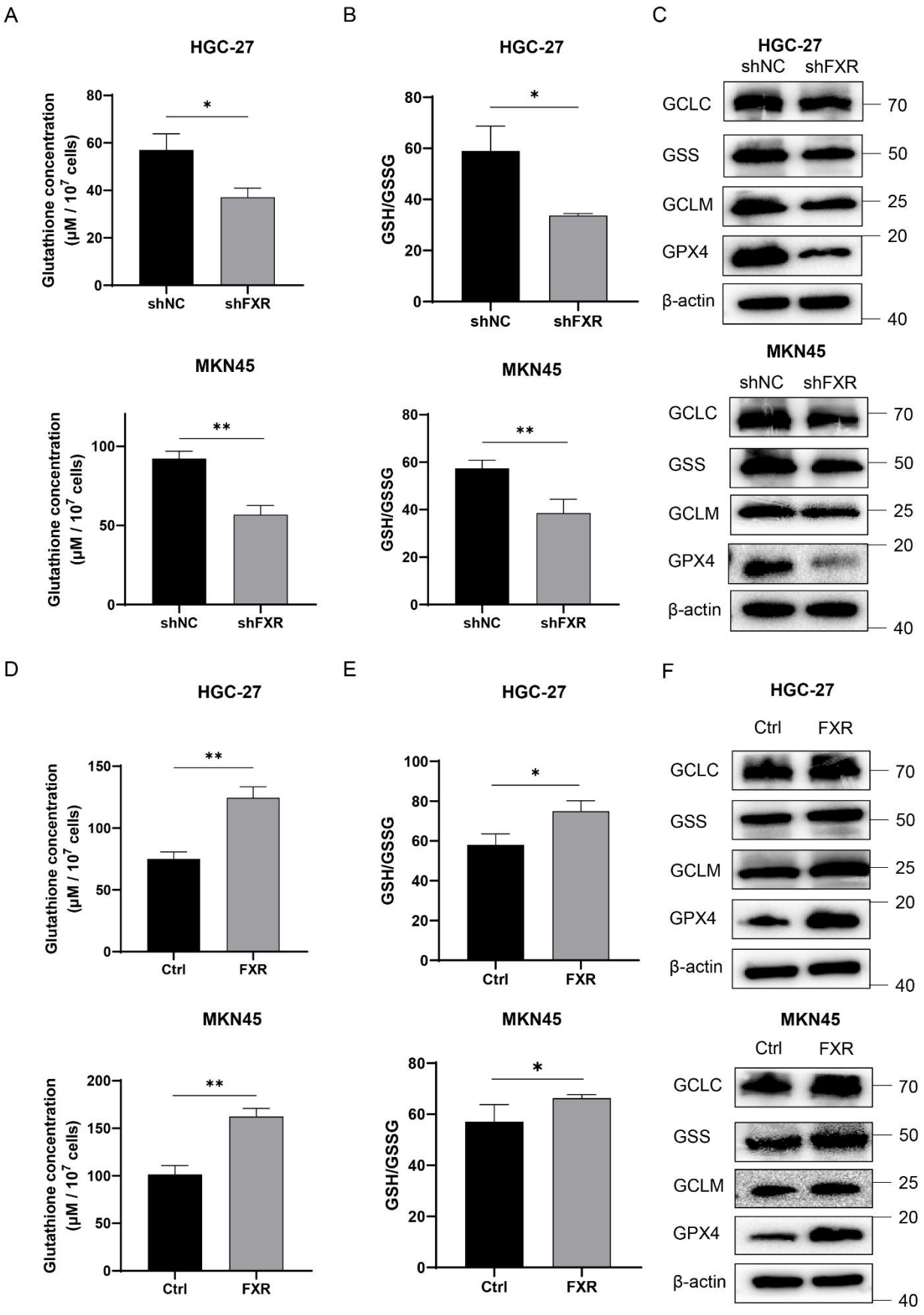
A
HGC-27
Glutathione concentration (μM / 10^7 cells)
shNC
shFXR
MKN45
B
HGC-27
GSH/GSSG
MKN45
C
HGC-27
shNC shFXR
GCLC
GSS
GCLM
GPX4
β-actin
70
50
25
20
40
MKN45
D
HGC-27
Ctrl
FXR
MKN45
E
HGC-27
MKN45
F
HGC-27
Ctrl FXR
MKN45

Figure 4FXR significantly promoted the synthesis of GSH and the level of GPX4 in GC cells. A, B: Alterations of GSH concentrations and the GSH/GSSG ratio in HGC-27 and MKN-45 cells transfected with the shNC or shFXR plasmid. C: Protein expression of GCLC, GSS, GCLM, and GPX4 in HGC-27 and MKN-45 cells transfected with the shNC or shFXR plasmid. D,E: Alterations of GSH concentrations and the GSH/GSSG ratio in HGC-27 and MKN-45 cells transfected with the control or FXR-coding plasmid. F: Protein expression of GCLC, GSS, GCLM, and GPX4 in HGC-27 and MKN-45 cells transfected with the control or FXR-coding plasmid. $*P<0.05$, $**P<0.01$, $***P<0.001$. These experiments were repeated three times. FXR: farnesoid X receptor; GSH: glutathione; GC: gastric cancer; GPX4: glutathione peroxidase 4; GSSG: oxidized glutathione ; GSH/GSSG: reduced glutathione to oxidized glutathione; NC: negative control.

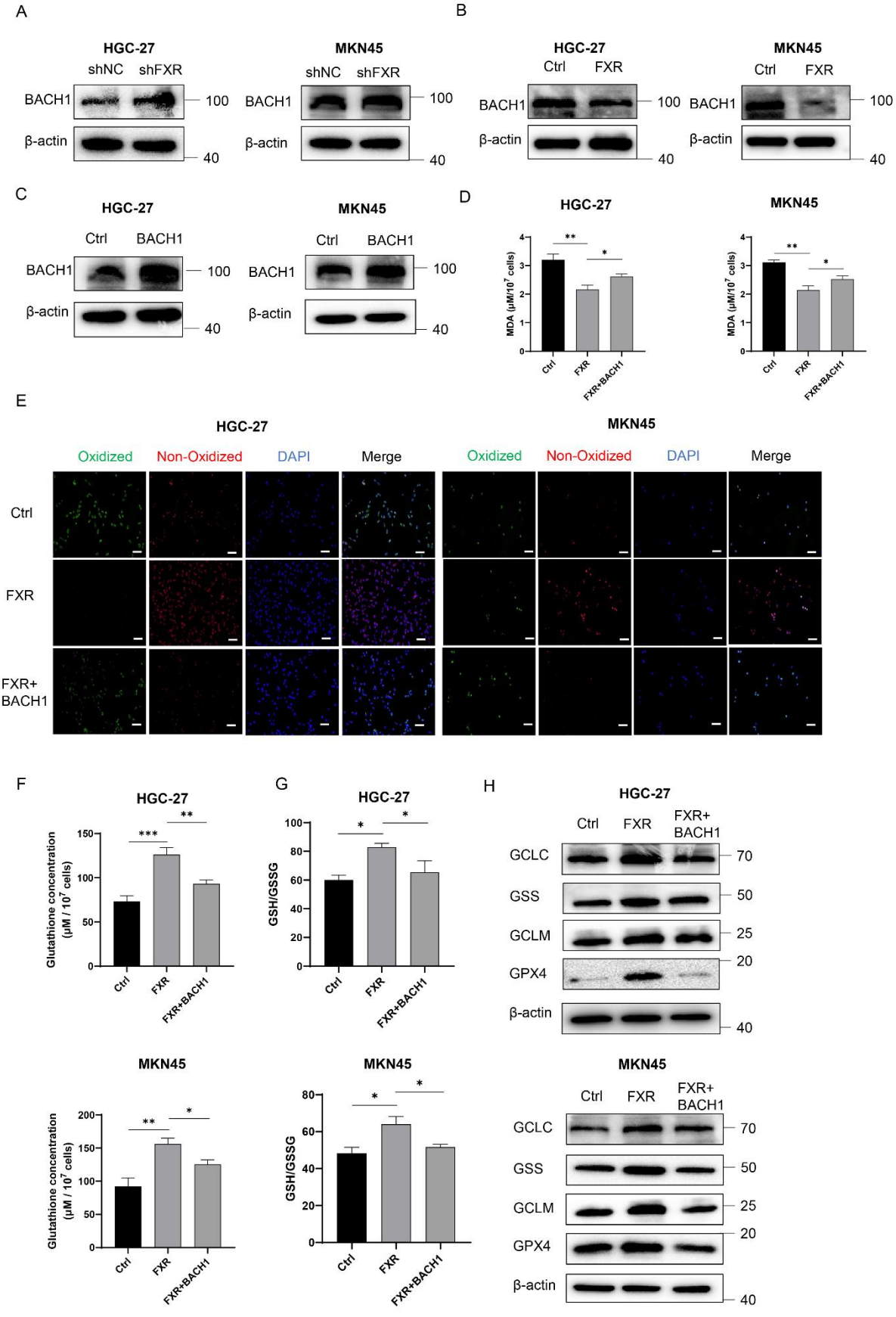
A
HGC-27
shNC
shFXR
BACH1
β-actin
100
40
MKN45
shNC
shFXR
BACH1
β-actin
100
40
B
HGC-27
Ctrl
FXR
BACH1
β-actin
100
40
MKN45
Ctrl
FXR
BACH1
β-actin
100
40
C
HGC-27
Ctrl
BACH1
BACH1
β-actin
100
40
MKN45
Ctrl
BACH1
BACH1
β-actin
100
40
D
HGC-27
MDA (μM/10^7 cells)
Ctrl
FXR
FXR+BACH1
MKN45
MDA (μM/10^7 cells)
Ctrl
FXR
FXR+BACH1
E
HGC-27
Oxidized
Non-Oxidized
DAPI
Merge
MKN45
Oxidized
Non-Oxidized
DAPI
Merge
Ctrl
FXR
FXR+ BACH1
F
HGC-27
Glutathione concentration (μM / 10^7 cells)
Ctrl
FXR
FXR+BACH1
MKN45
Glutathione concentration (μM / 10^7 cells)
Ctrl
FXR
FXR+BACH1
G
HGC-27
GSH/GSSG
Ctrl
FXR
FXR+BACH1
MKN45
GSH/GSSG
Ctrl
FXR
FXR+BACH1
H
HGC-27
Ctrl
FXR
FXR+ BACH1
GCLC
GSS
GCLM
GPX4
β-actin
70
50
25
20
40
MKN45
Ctrl
FXR
FXR+ BACH1
GCLC
GSS
GCLM
GPX4
β-actin
70
50
25
20
40

Figure 5FXR exerted anti-ferroptosis effects by inhibiting BACH1. A: Protein expression of BACH1 in GC cells transfected with the shNC or shFXR plasmid for 24 hours. B: WB analysis of BACH1 protein expression in GC cells transfected with the shNC or shFXR plasmid for 24 hours. C: HGC-27 and MKN-45 cells were transfected with the control or BACH1-coding plasmid and confirmed through WB analysis. D, E: MDA production and BODIPY-589/591 C11 staining of GC cells after transfection with the FXR-coding plasmid together with or without the BACH1-coding plasmid and erastin treatment (5 μM) for 24 hours. F, G: Alterations of GSH concentrations and the GSH/GSSG ratio in HGC-27 and MKN-45 cells after transfection with the FXR-coding plasmid together with or without the BACH1-coding plasmid. H: WB analysis of GCLC, GSS, GCLM, and GPX4 protein expression after transfection with the FXR-coding plasmid together with or without the BACH1-coding plasmid. Scale bar, 100 μm. $*P<0.05$, $**P<0.01$, $***P<0.001$. These experiments were repeated three times. FXR: farnesoid X receptor; BACH1: BTB and CNC homology1; GC: gastric cancer; NC: negative control; WB: western blot; MDA: malondialdehyde.

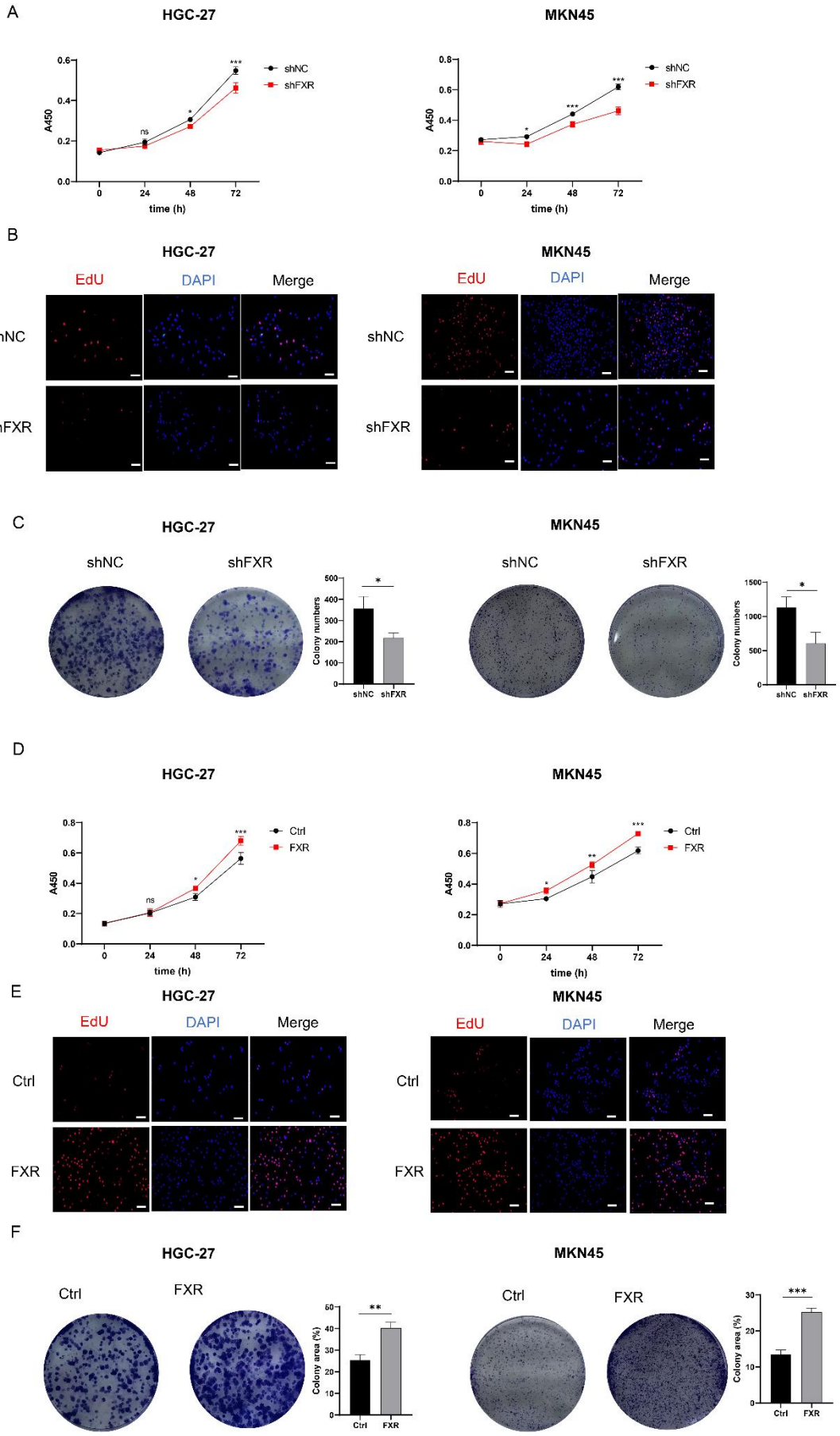
A
HGC-27
MKN45
shNC
shFXR
A450
time (h)
B
EdU
DAPI
Merge
C
Colony numbers
D
Ctrl
FXR
E
F
Colony area (%)

Figure 6FXR promoted proliferation of GC cells. Malignant proliferation assays, including (A) cell viability, (B) Edu staining, and (C) colony formation assays, were performed in GC cells after transfection with the shNC or shFXR plasmid. (D) Cell viability, (E) Edu staining, and (F) colony formation assays were performed in GC cells after transfection with the control or FXR-coding plasmid. Scale bar, 100 μm. $*P<0.05$, $**P<0.01$, $***P<0.001$. These experiments were repeated three times. FXR: farnesoid X receptor; GC: gastric cancer; Edu: 5-ethynyl-2′-deoxyuridine; NC: negative control.