

Gastrin Enhances Autophagy and Promotes Gastric Carcinoma Proliferation via Inducing AMPK α

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Gastric cancer (GC) is one of the most frequent epithelial malignancies worldwide. The gastrointestinal (GI) peptide gastrin is an important regulator of the secretion and release of gastric acid from stomach parietal cells, and it also plays a vital role in the development and progression of GC. The aim of the current study was to investigate the role and underlying mechanism of gastrin and autophagy in regulating GC tumorigenesis. Gastrin-17 amide (G-17) was applied in the GC cell lines SGC7901 and MGC-803. The results showed that G-17 maintained the high viability of SGC7901 and MGC-803. The expression of autophagy marker proteins LC3II and Beclin1 was significantly increased, while the autophagy substrate p62 was obviously decreased in the gastrin group compared with the control group. Moreover, G-17 strengthened the expressions of AMPK α , Ras, Raf, MEK, and ERK1/2. Additionally, administration of AMPK α siRNA counteracted the effect of gastrin in SGC7901 cells. Finally, in an *in vivo* study of the tumor growth and survival rate of rats, the levels of AMPK α /Ras/Raf/MEK/ERK were significantly increased in the gastrin group and decreased following AMPK α shRNA injection. In conclusion, these findings indicate that gastrin plays a tumorigenic role by promoting autophagy in GC and may provide a novel therapeutic target for GC treatment.

Key words: Gastric cancer (GC); Gastrin; Autophagy; AMP-activated protein kinase (AMPK α)

INTRODUCTION

Gastric cancer (GC) is one of the main causes of cancer-related deaths and one of the most frequent epithelial malignancies worldwide, especially in China and Japan¹. Despite the advances made in the treatment and prevention of GC, the survival rate for this cancer has not been significantly improved². The development and progression of GC involve multiple steps and various mechanisms. Autophagy is a highly conserved intracellular homeostatic pathway in humans. It has been reported that the autophagic process provides energy to cells and prevents the accumulation of toxins under nutrient-depleted conditions or under various cellular stresses³. Thus, autophagy benefits tumor cell survival by serving as a self-defensive mechanism in tumorigenesis under extraordinary circumstances⁴. Growing evidence has revealed the important role of autophagy in different physiopathological processes of diseases, including vascular disorders⁵, neurodegenerative diseases⁶, and cancers⁷. To date, the potential clinical significance of autophagy-related therapeutic strategies has not been fully investigated. Thus, it is vital to understand

and develop new agents that regulate autophagy and improve the disease management of GC.

The gastrointestinal (GI) peptide gastrin, which is secreted by G cells in the mucosal membrane and the antrum of the stomach, is an important regulator of the secretion and release of gastric acid from stomach parietal cells⁸. The posttranslation processing of gastrin is from the gastrin precursor preprogastrin, which gives rise to various products including glycine-extended gastrin-34 (gly-G-34), amidated gastrin-34 (G-34), progastrin (pro-G), glycine-extended gastrin-17 (gly-G-17), and amidated gastrin-17 (G-17)⁹. It has been reported that G-17 accounts for over 90% of all gastrin secreted by G cells in most mammals¹⁰. Additionally, research has shown that G-17 plays an important role in the genesis and development of numerous carcinomas such as colon, pancreatic, non-small cell lung, and GCs¹¹. Gastrin and cholecystokinin (CCK) is one of the unique growth-related receptors that stimulate tumor proliferation. Our previous study demonstrated that cholecystokinin-B (CCK-B) promotes the metastasis of GC after binding with its ligand gastrin¹².

However, the effect of CCK-B/gastrin in cell proliferation, apoptosis, and other metastases of GC has not been clearly understood.

A recent study reported that gastrin intervention elevated the expression of salt-inducible kinase 1 (SIK1/Snf1lk), which belongs to the AMP-activated protein kinase (AMPK) family of kinases¹³. It is well known that the AMPK family of kinases plays a major role in regulating metabolism, cell growth, and autophagy. A recent study reported that AMPK promoted the Ras (rat sarcoma viral oncogene)/Raf pathway by activating autophagy in cancer cells¹⁴, which led to increases in cell adhesion, proliferation, and so on¹⁵. These studies indicate the need for further research into gastrin to understand the underlying mechanism of gastrin and autophagy in regulating GC tumorigenesis.

In the present study, we aimed to identify the role of gastrin in the progression of GC. We found that gastrin promoted autophagy in GC cell lines. In addition, gastrin increased GC cell proliferation by elevating the AMPK and Ras pathways. Taken together, our results suggest that gastrin enhances the autophagic process and increases cell proliferation in GC and may provide a novel therapeutic target for improving cancer survival.

MATERIALS AND METHODS

Ethical Approval

Our study was approved by the ethics committee of the Experimental Animal Center of The Second Affiliated Hospital of Xi'an Jiaotong University.

Cell Lines and Cultures

The human GC cell lines SGC7901 and MGC-803 were purchased from Bioleaf Biotech (Shanghai, P.R. China). Cell culture plates were precovered with 5 $\mu\text{g}/\text{cm}^2$ Matrigel (BD Biosciences, Shanghai, P.R. China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS supplemented with 100 mg/ml streptomycin and 100 U/ml penicillin at 37°C with 5% CO₂. Cells were randomly divided into four groups: control group (normal SGC7901 or MGC-803 cells without any treatment), the gastrin group [cells incubated with 1 $\times 10^{-7}$ mol/L G-17 (Aphtron Corp., Woodland, CA, USA), the proglumide (PGL) group [cells incubated with 1 $\times 10^{-7}$ mol/L PGL (a receptor antagonist of G-17; Santa Cruz Biotechnology, Santa Cruz, CA, USA), which is a gastrin receptor antagonist], and the gastrin+PGL group (cells incubated with 1 $\times 10^{-7}$ mol/L G-17 and 1 $\times 10^{-7}$ mol/L PGL at the same time).

Cell Apoptosis Analysis

To measure the apoptosis rate of cells, we used the flow cytometry method. Cells were harvested, washed

with PBS three times, and then fixed with 75% ethanol overnight at 4°C. After incubation with RNase at 37°C for 30 min, cells were stained with propidium iodide for 30 min. A total of 10⁴ events were examined by a FACSCalibur flow cytometer (Becton-Dickinson, Shanghai, P.R. China). The histograms were analyzed by the CellQuest software (Becton-Dickinson, Mountain View, CA, USA). The results are presented as the percentage of cells in each phase.

MTT Assay

Cell viability was detected using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were first seeded into 96-well plates at a density of 6 $\times 10^3$ cells/well. The surviving fractions were detected at 0, 24, 48, 72, 96, and 120 h, respectively. Thereafter fresh medium containing 5 mg/ml MTT (Sangon, Shanghai, P.R. China) was added and incubated for 4 h after the old medium was discarded. Finally, cell viability was measured using a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) at 470 nm.

Western Blot Analysis

The preparation of cell extracts and the following Western blotting were performed according to the standard instructions. Briefly, a total of 25 μg of proteins was loaded and then separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then electrotransferred to nitrocellulose membranes (Amersham, Little Chalfont, UK). The membranes were blocked with 3% nonfat milk for 1 h at 37°C. After washing with Tris-buffered saline with Tween (TBST) three times, the membranes were then incubated with primary antibodies against (microtubule-associated protein 1 light chain 3) LC3I/II, Beclin1, p62, AMPK α , Ras, Raf, MEK, ERK1/2, and β -actin (Santa Cruz Biotechnology) at 4°C overnight. The peroxidase-conjugated secondary antibody (1:1,000; Boster Corporation, Wuhan, Hubei, P.R. China) was then used and incubated for 1 h at room temperature. The immunoreactive protein bands were then observed using an enhanced chemiluminescence detection system (Amersham). The expression level of proteins was normalized to β -actin.

AMPK α Silencing

In order to confirm the role of the AMPK α pathway on the effect of gastrin in GC cells, we transfected a small interfering RNA (siRNA) to silence the expression of AMPK α in SGC7901 cells. Cells were divided into five groups: control group (cells without any treatment), gastrin group (cells treated with 1 $\times 10^{-7}$ mol/L G-17), siRNA control group [cells transfected with siRNA control (Shanghai GenePharma, Shanghai, P.R.

China) for 48 h and then incubated with 1×10^{-7} mol/L G-17], siAMPK α group [cells transfected with AMPK α siRNA (Shanghai GenePharma) for 48 h and then incubated with 1×10^{-7} mol/L G-17], and LY294002 group (cells treated with the autophagy inhibitor LY294002 at 50 μ M and 1×10^{-7} mol/L G-17).

Gastric Cancer Xenografts

All 27 6-week-old female severe combined immunodeficient (SCID) mice (Animal Center of Xi'an Jiaotong University, Xi'an, P.R. China) were housed and manipulated according to the protocols approved by the Experimental Animal Center of The Second Affiliated Hospital

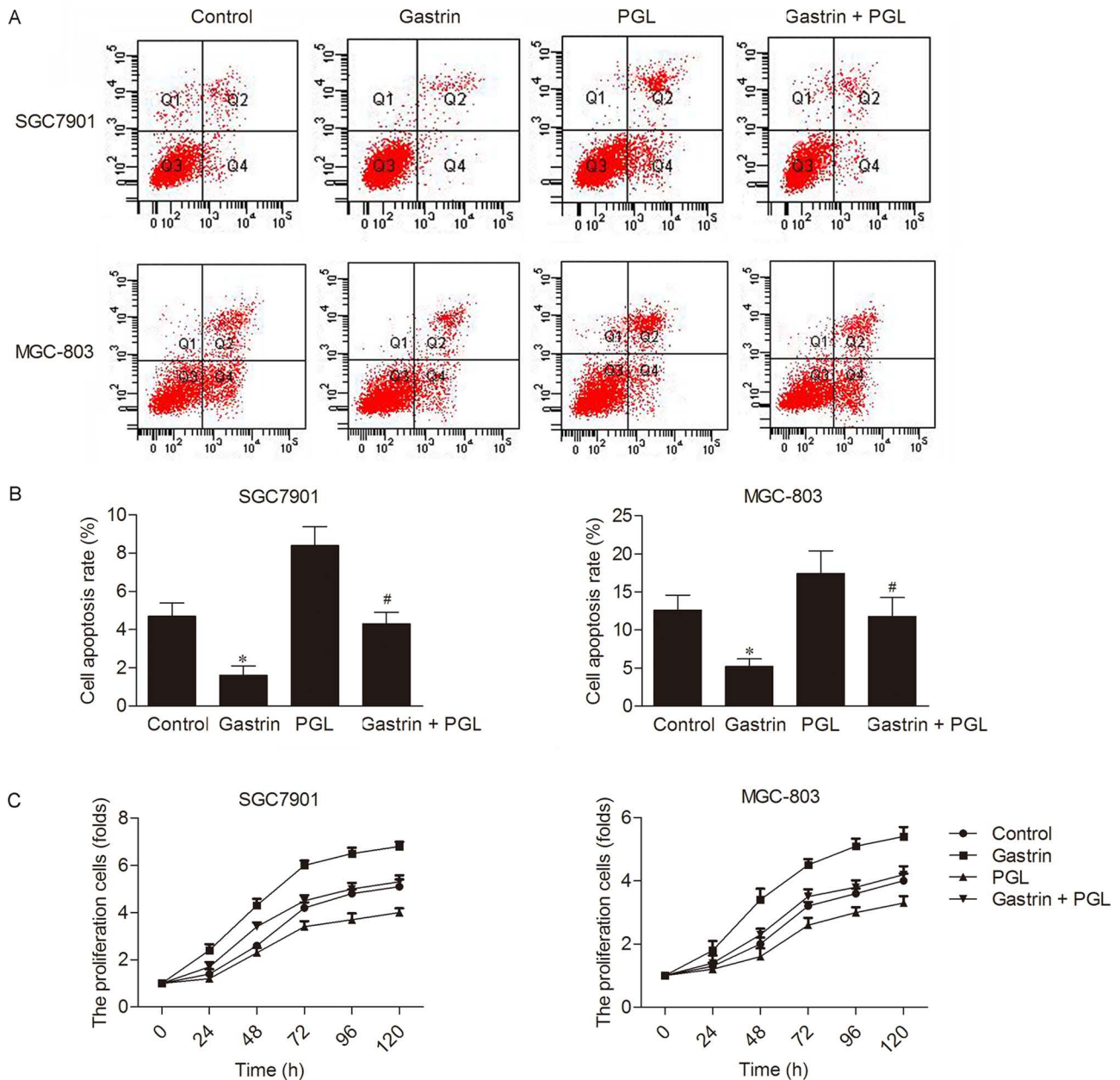


Figure 1. Gastrin maintains the high viability of SGC7901 and MGC-803 cells via binding to cholecystokinin-B (CCK-B). Control group: cells without any treatment; gastrin group: cells incubated with 1×10^{-7} mol/L G-17; PGL group: cells incubated with 1×10^{-7} mol/L PGL; gastrin+PGL group: cells incubated with G-17 (1×10^{-7} mol/L) and PGL (1×10^{-7} mol/L) at the same time. (A, B) Cell apoptosis of SGC7901 and MGC-803 was measured by flow cytometry. (C) The proliferation of SGC7901 and MGC-803 cells was detected at the time points 24, 48, 72, 96, and 120 h by MTT assays. All experiments were repeated three times. * $p < 0.05$ versus control group; # $p < 0.05$ versus PGL group.

of Xi'an Jiaotong University. Different SGC7901 cells were injected subcutaneously into the right scapula of each experimental mouse: control group (SGC7901 cells without any treatment), gastrin group (cells incubated with 1×10^{-7} mol/L G-17), and siAMPK α group (SGC7901 cells transfected with AMPK α siRNA for 48 h and then incubated with 1×10^{-7} mol/L G-17). Tumor volume was measured using a caliper and monitored every 5 days. After 30 days of inoculation, the mice were sacrificed by subcutaneous injection with sodium pentobarbital (100 mg/kg). A portion of each tumor was selected for Western blotting.

Statistical Analysis

All results in this study were presented as mean \pm SD from a minimum of three replicates. All statistical analyses were evaluated by SPSS version 15.0 statistical software (Chicago, IL, USA). Student's *t*-test was used when comparing only two groups; one-way ANOVA was used when more than two groups were compared. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Gastrin Maintains the High Viability of SGC7901 and MGC-803 Cells via Binding to CCK-B

To investigate the impact of gastrin on the survival of GC cells, we explored the apoptosis and proliferation of SGC7901 and MGC-803 cells induced by gastrin. Flow cytometric analysis indicated that gastrin strongly suppressed the apoptosis rate compared with the control group, while the addition of PGL in the gastrin group increased the apoptosis of cells ($p < 0.05$) (Fig. 1A and B). The results of the MTT analysis indicated that the growth of SGC7901 and MGC-803 cells was enhanced significantly by gastrin but decreased following the addition of PGL in the gastrin group ($p < 0.05$) (Fig. 1C). These results suggested that by binding to CCK-B, gastrin can maintain the high viability of SGC7901 and MGC-803 cells.

Gastrin Enhances the Autophagy of SGC7901 Cells

Given that gastrin sustains the survival of GC cells, we next tested whether gastrin played a functional role in autophagy. SGC7901 cells were chosen for the following experiments. Western blot analysis indicated that LC3II and Beclin1 expression levels were elevated significantly in the gastrin group compared with the control group, and PGL partly inhibited the overexpression of LC3II and Beclin1 induced by gastrin ($p < 0.05$) (Fig. 2A and B). Consistent with this, the level of autophagy substrate p62 was decreased significantly in the gastrin group in comparison with the control group, and PGL partly restored the low level of p62 caused by gastrin. Additionally, the ratio of LC3II/LC3I was significantly increased in the

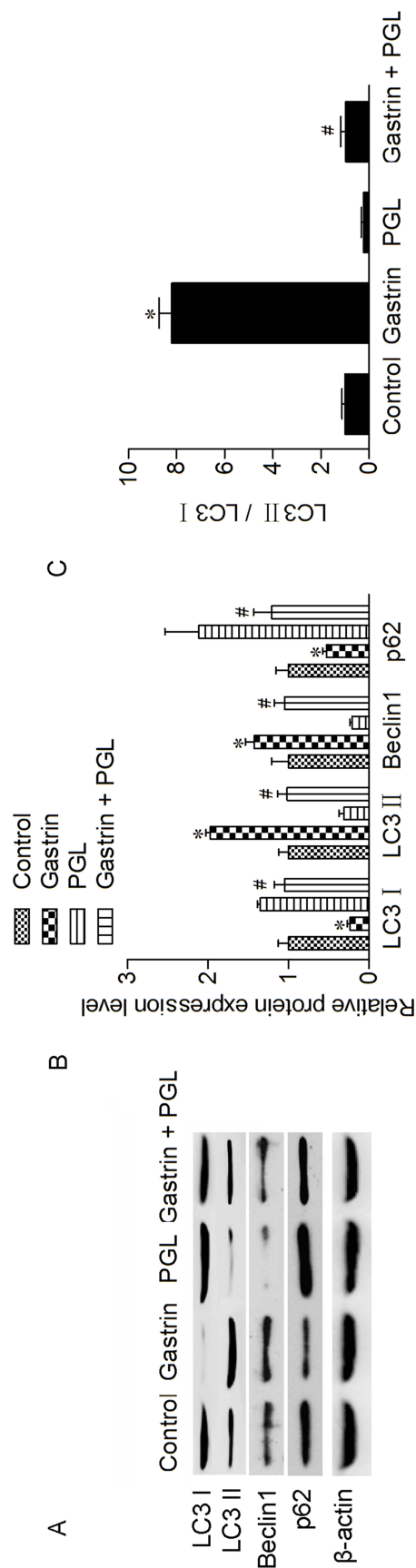


Figure 2. Gastrin enhances the autophagy of SGC7901 cells. (A) The expression of LC3II, LC3I, Beclin1, and p62 was detected in the SGC7901 cells by Western blotting. (B, C) Relative protein expression levels were quantified using the Image-Pro Plus 6.0 software and normalized to β -actin. Data are presented as the mean \pm SD of all three experiments. * $p < 0.05$ versus control group; # $p < 0.05$ versus PGL group.

gastric group and decreased with PGL administration (Fig. 2C). These results suggested that gastrin enhances the autophagy of SGC7901 cells.

Gastrin Strengthens the Expressions of AMPK α and Ras/Raf/MEK/ERK Pathway Proteins

Autophagy has been reported to be mediated by the cellular energy sensor AMPK, which allows cancer tolerance to Ras/Raf pathway inhibitors. We further explored the mechanism of gastrin in the autophagy of SGC7901 cells. Western blot analysis indicated that the expressions of AMPK α , Ras, Raf, MEK, and ERK1/2 were upregulated by gastrin compared with the control group and were downregulated by adding PGL in the gastrin group (Fig. 3). These results illustrated that gastrin enhances the expressions of AMPK α and Ras/Raf/MEK/ERK pathway proteins via binding to CCK-B.

AMPK α Is Involved in Gastrin Promoting Proliferation and Autophagy in SGC7901 Cells

To confirm the above result, a siRNA specific for AMPK α and autophagy inhibitor LY294002 were applied with gastrin in SGC7901 cells. The cell growth was strongly inhibited after the silencing of AMPK α or the addition of LY294002 compared with the gastrin group (Fig. 4A). The expression of autophagy markers exhibited no significant difference between the gastrin group and the siRNA control group. However, the interference of the addition of AMPK α or LY294002 effectively suppressed the level of LC3II and Beclin1 and elevated the level of p62 in the siAMPK α group compared with the gastrin group. These results indicated that gastrin promoted the proliferation and autophagy of SGC7901 cells by inducing AMPK α .

Gastrin Accelerates Gastric Cancer Growth In Vivo via Inducing AMPK α

To further investigate the effect of gastrin on tumor growth in vivo, SGC7901 cells treated with gastrin alone or with AMPK α siRNA were injected into the right scapula of each mouse ($n=9$). The tumor grew more slowly in the siAMPK α group compared with mice in the gastrin group (Fig. 5A), and mice in the siAMPK α group exhibited significantly smaller tumor volume compared with the gastrin group (Fig. 5B). The interference of AMPK α effectively reversed the level of autophagy markers (Fig. 5C and D) and AMPK α /Ras/Raf/MEK/ERK (Fig. 5E and F) in the siAMPK α group compared with the gastrin group. These results indicated that gastrin accelerates GC growth in vivo via inducing AMPK α .

DISCUSSION

Gastrin is a crucial regulatory peptide hormone that plays an important role in the integration of endocrine and exocrine functions in the GI tract. It has been reported that gastrin regulates various cellular processes in the gastric epithelium and in carcinoma cells including proliferation, migration, invasion, and antiapoptosis^{16,17}. A recent study suggested that gastrin elevated the expression of SIK1, which is a member of the AMPK family of kinases, and participated in the activation of autophagy¹³. Our previous research demonstrated that gastrin promoted the metastasis of GC cells and suggested that gastrin may be a potential therapeutic target for the treatment of GC¹². However, the connection and underlying mechanism of gastrin and autophagy in GC have not been investigated.

The CCK-B receptor, which is generally distributed all over the human GI tract and located on the basolateral

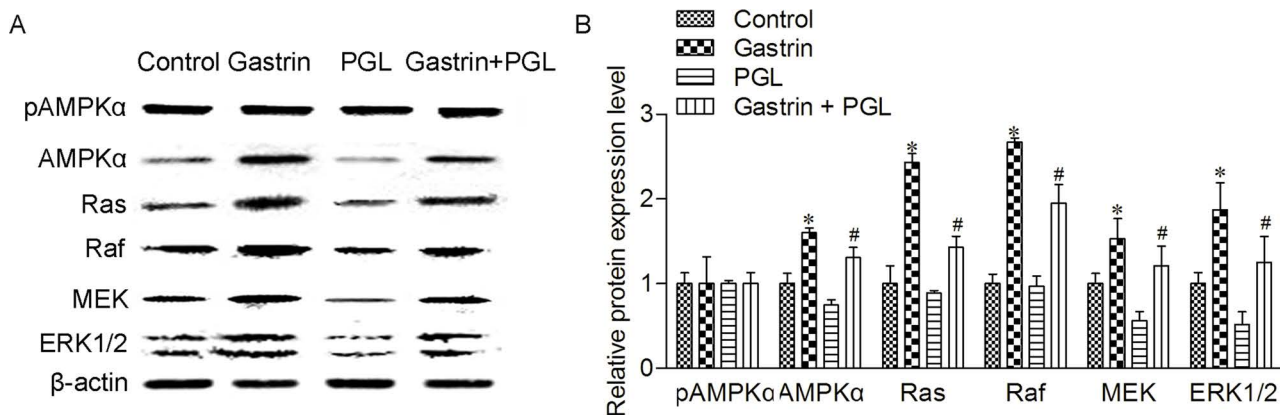


Figure 3. Gastrin strengthens the expressions of AMPK α and Ras/Raf/MEK/ERK pathway proteins. (A) The expression of pAMPK α , AMPK α , Ras, Raf, MEK, and ERK1/2 was measured in the SGC7901 cells by Western blotting. (B) Relative protein expression levels were quantified using the Image-Pro Plus 6.0 software and normalized to β -actin. Data are presented as the mean \pm SD of all three experiments. * $p < 0.05$ versus control group; # $p < 0.05$ versus PGL group.

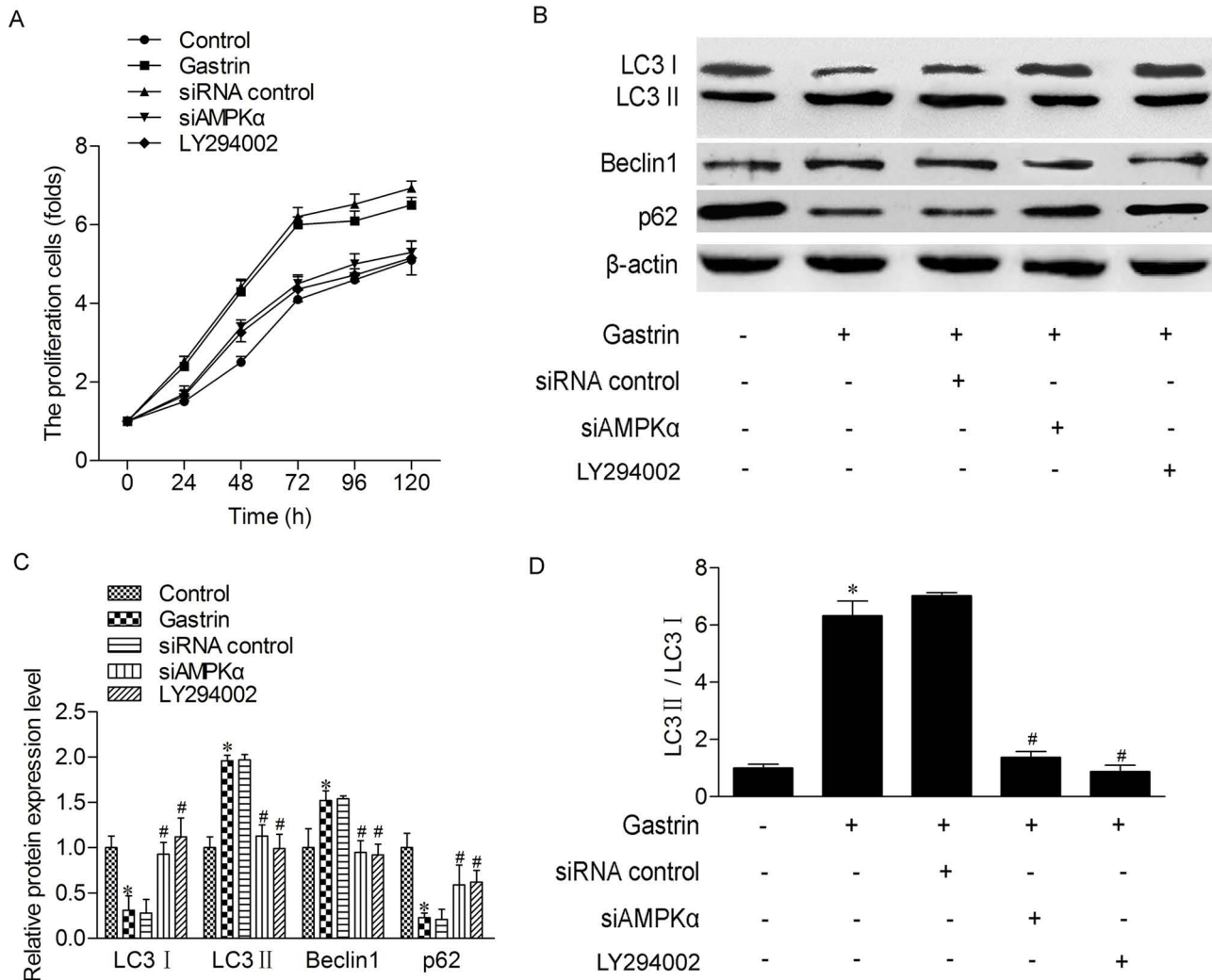


Figure 4. AMPK α is involved in gastrin promoting proliferation and autophagy in SGC7901 cells. Control group: cells without any treatment; gastrin group: cells incubated with 1×10^{-7} mol/L G-17; siRNA control group: cells transfected with siRNA control for 48 h and then incubated with 1×10^{-7} mol/L G-17; siAMPK α group: cells transfected with AMPK α siRNA for 48 h and then incubated with 1×10^{-7} mol/L G-17; LY294002 group: cells treated with 50 μ M LY294002 and 1×10^{-7} mol/L G-17. (A) The proliferation of SGC7901 cells detected at the time points 24, 48, 72, 96, and 120 h by MTT assays. (B) The expression of LC3II, LC3I, Beclin1, and p62 was detected in the SGC7901 cells by Western blotting. (C, D) Relative protein expression levels were quantified using the Image-Pro Plus 6.0 software and normalized to β -actin. Data are presented as the mean \pm SD of all three experiments. * $p < 0.05$ versus control group; # $p < 0.05$ versus gastrin group.

domain of the cell membrane, is known to be immunoreactive and mediates the physiological function of gastrin. Research has demonstrated the proproliferation effect of gastrin on various malignancies, including colorectal cancer and GC, by binding with the CCK-B receptor¹⁸. To investigate the function of gastrin and its receptor CCK-B in GC, we used the gastrin receptor antagonist PGL. The results illustrated that the high viability of GC cells was enhanced with gastrin administration, but inhibited after the addition of PGL in two GC cell lines, SGC7901 and MGC-803. This result is consistent with the study of

Zhai et al. and confirmed the proproliferation effect of gastrin, which functioned by binding to CCK-B in GC cells¹⁹. A study of gastrin receptor antagonists reported that gastrin not only functioned on cell proliferation but also influenced cell apoptosis in GC cells²⁰. Consistently, the antiapoptotic effect of gastrin/CCK-B on GC cells was observed in this study. These results indicated the vital role of gastrin on the proliferation and apoptosis in GC cells via binding to the CCK-B receptor.

Autophagy is known as a highly regulated catabolic pathway, which is responsible for the degradation of

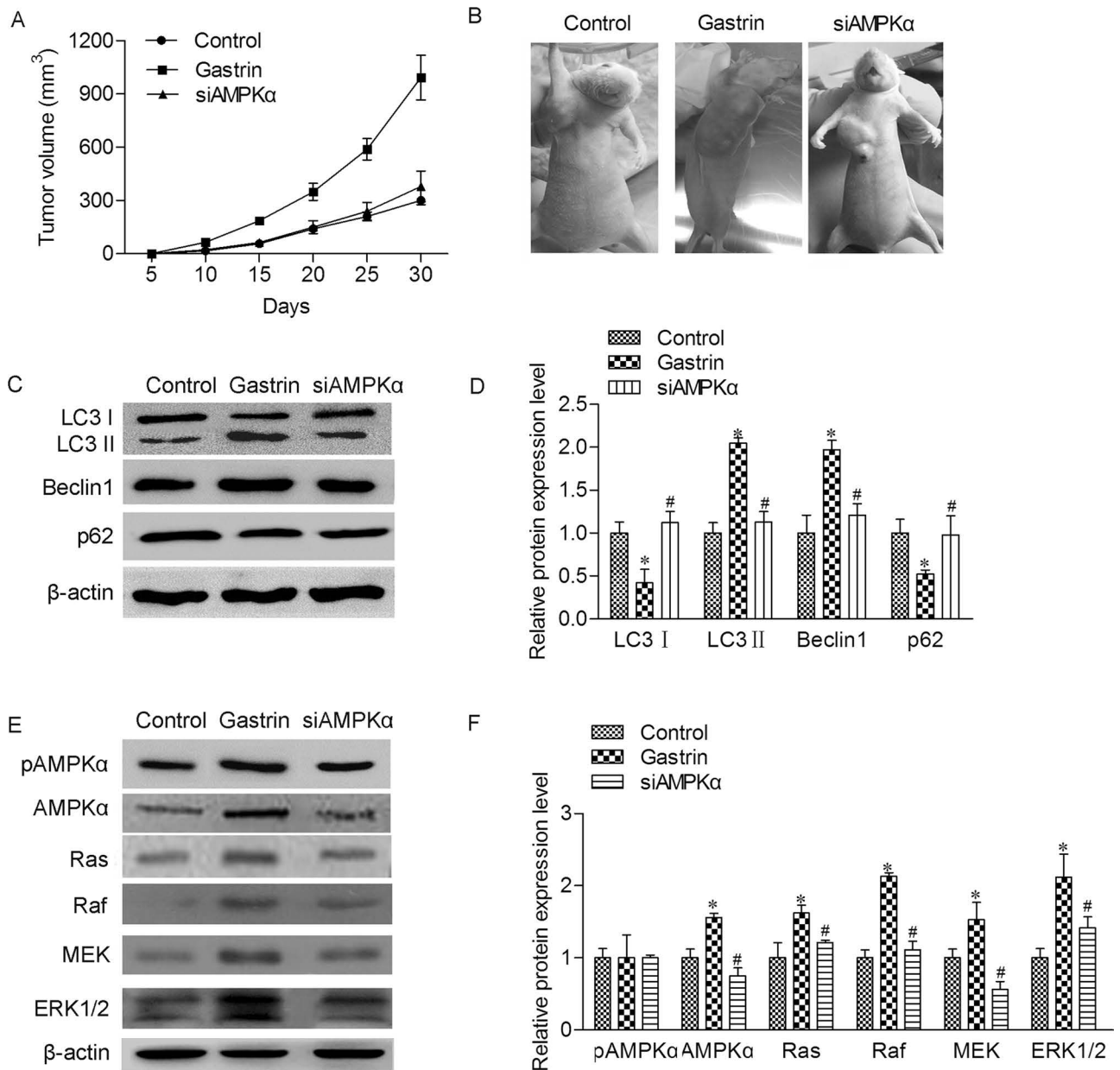


Figure 5. Gastrin accelerates gastric cancer growth in vivo via inducing AMPK α . Control group: severe combined immunodeficient (SCID) mice injected with SGC7901 cells; gastrin group: SCID mice injected with 1×10^{-7} mol/L G-17-incubated SGC7901 cells; siAMPK α group: SGC7901 cells transfected with AMPK α siRNA and incubated with 1×10^{-7} mol/L G-17, and then injected into SCID mice. (A) The tumor growth curve of SCID mice. (B) The representative tumor of SCID mice among different groups. (C, E) The protein expression was measured by Western blot. (D, F) Relative protein expression levels were quantified using the Image-Pro Plus 6.0 software and normalized to β -actin. Data are presented as the mean \pm SD of all three experiments. * $p < 0.05$ versus control group; # $p < 0.05$ versus gastrin group.

damaged intracellular organelles and long-lived proteins³. It has been reported that autophagy is perturbative and benefits cell survival in GC⁴. The progression of autophagy involves the assembly of autophagosomes, which is regulated by the conversion of cytosolic LC3I into the lipidated form of LC3II²¹. Therefore, the levels

of LC3I and LC3II in cells represent the appearance of autophagic response. The expression of LC3II, along with the ratio of LC3II/LC3I, was promoted by gastrin stimulation and reversed by PGL administration in the GC cell line SGC7901. This result suggested that the autophagy response was activated by gastrin, which is consistent

with the study by Selvik et al.¹³. Beclin1 is a component of the phosphoinositide 3-kinase (PI3K) complex and contributes to the biosynthesis of autophagosomes²². p62 is a ubiquitin-binding protein and serves as a selective autophagy substrate²³. Beclin1 and p62 were widely used as autophagy markers in numerous studies and have been reported to be upregulated in GC^{24,25}. The expression of Beclin1 was increased, while the expression of p62 decreased in gastrin-treated GC cells in our study, further confirming the activation of autophagy. These results suggested that gastrin/CCK-B stimulates the autophagy response in GC cells.

It has been reported that the AMPK pathway is essential in the regulation of cell survival, cell death, and energy homeostasis²⁶. A study indicated that gastrin stimulated the AMPK pathway, leading to the activation of autophagy in gastric adenocarcinoma cells¹³. Another study suggested that AMPK promotes the RAS/Raf/MEK pathway to activate autophagy and inhibit the carcinogenesis function of Ras²⁷. The activation of ERK1/2, the downstream gene of RAS/Raf/MEK, is closely related to the accumulation of autophagosomes. In the present study, the expression of the AMPK pathway proteins, including AMPK α , RAS, Raf, MEK, and ERK1/2, was significantly increased by the addition of gastrin but were inhibited by PGL in GC cell line SGC7901. Additionally, AMPK α siRNA transfection reversed the proliferation and autophagy activation effect of gastrin in SGC7901 cells. These results provide further evidence of the autophagy-promoting role of gastrin in GC via promoting the AMPK α /RAS/Raf/MEK/ERK1/2 pathway. LY294002 is widely used as an autophagy inhibitor in various cell studies²⁸. Our results demonstrated that the effect of LY294002 was similar to AMPK α siRNA transfection in gastrin-treated SGC7901 cells, which further confirmed the effect of gastrin on autophagy in GC. Moreover, the in vivo study illustrated that the addition of gastrin increased tumor growth and activated autophagy in mice, while the silencing of AMPK α reversed this effect. Taken together, we suggest that gastrin promotes GC progression and enhances autophagy by activating the AMPK α /RAS/Raf/MEK/ERK1/2 pathway.

In summary, gastrin promoted GC metastasis in our previous study and stimulated tumor growth via activating autophagy in the current study. These findings suggested that gastrin may serve as a potential therapeutic target in GC. Because of the effects of gastrin, we may identify a new avenue based on gastrin for GC treatment.

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