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Over-Expression of Gastrin Inhibits Apoptosis of Gastric Cancer Cells via Reactive Oxygen Species and Annexin A2-Mediated Mitochondrial Dysfunction

La Sobreexpresión de Gastrina Inhibe la Apoptosis de las Células de Cáncer Gástrico a Través de Especies Reactivas de Oxígeno y Disfunción Mitocondrial Mediada por Anexina A2

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SUMMARY: Gastrin plays a vital role in the development and progression of gastric cancer (GC). Its expression is up-regulated in GC tissues and several GC cell lines. Yet, the underlying mechanism remains to be investigated. Here, we aim to investigate the role and mechanism of gastrin in GC proliferation. Gastrin-overexpressing GC cell model was constructed using SGC7901 cells. Then the differentially expressed proteins were identified by *iTRAQ* analysis. Next, we use flow cytometry and immunofluorescence to study the effect of gastrin on the mitochondrial potential and mitochondria-derived ROS production. Finally, we studied the underlying mechanism of gastrin regulating mitochondrial function using Co-IP, mass spectrometry and immunofluorescence. Overexpression of gastrin promoted GC cell proliferation *in vitro* and *in vivo*. A total of 173 proteins were expressed differently between the controls and gastrin-overexpression cells and most of these proteins were involved in tumorigenesis and cell proliferation. Among them, Cox17, Cox5B and ATP5J that were all localized to the mitochondrial respiratory chain were down-regulated in gastrin-overexpression cells. Furthermore, gastrin overexpression led to mitochondrial potential decrease and mitochondria-derived ROS increase. Additionally, gastrin-induced ROS generation resulted in the inhibition of cell apoptosis via activating NF-κB, inhibiting Bax expression and promoting Bcl-2 expression. Finally, we found gastrin interacted with mitochondrial membrane protein Annexin A2 using Co-IP and mass spectrometry. Overexpr ession of gastrin inhibits GC cell apoptosis by inducing mitochondrial dysfunction through interacting with mitochondrial protein Annexin A2, then up-regulating ROS production to activate NF-κB and further leading to Bax/Bcl-2 ratio decrease.

KEY WORDS: Gastrin, gastric cancer; Mitochondria; ROS; NF-κB; Annexin A2.

INTRODUCTION

Gastrin, a peptide hormone produced primarily by antral gastrin expressing cells in the normal gastric antrum, was first identified by J.S Edkins in 1905 as an acid secretagogue (Engevik *et al*., 2020). Subsequent studies have confirmed that gastrin is an important physiological regulator of gastric acid secretion in the stomach and a regulator of gastric epithelial cell proliferation (Todisco *et al*., 2015; Sheng *et al*., 2020). However, in the past 20 years, the concept of gastrin as a simple gastrointestinal hormone has undergone major changes. Many studies have shown that gastrin might also act as a pro-survival factor in tumors, promoting tumor proliferation, angiogenesis, and migration, and inhibiting tumor cell apoptosis (Maddalo *et al*., 2014; Hayakawa *et al*., 2016; Smith *et al*., 2016; Sundaresan *et al*., 2017). Up-regulation of gastrin has been reported in colorectal cancer (Pagliarani & Gambino, 2019), pancreatic cancer (Al Menhali *et al*., 2017) malignant ovarian tumor (Evan & Vousden, 2001; Yin *et al*., 2010), glioblastoma (Friis-Hansen, 2007), and especially gastric cancer (GC) (Uehara *et al*., 2007). The study using gastrin over-expressing mice has identified the important role of gastrin in the growth of gastric mucosa (Haruma *et al*., 2018). Growth-promoting effects of gastrin on human gastric cancer cells have also been demonstrated in vitro and in vivo (Watson *et al*., 2006). However, the molecular pathway by which gastrin regulates tumorigenesis and cell apoptosis of GC have not been elucidated clearly.

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Mitochondrion plays a critical role in many cellular processes, such as cell death, autophagy and metabolic pathways (Pan *et al*., 2011; Iommarini *et al*., 2017). Mitochondrion has been implicated in carcinogenesis since the 1956 when Otto Warburg suggested that 'respiration damage' was a pivotal feature of cancer cells (Czarnecka *et al*., 2010; Visvader, 2011). Subsequently, a large numbers of studies show a decrease of mitochondrial respiration and oxidative phosphorylation in cancer (Lu *et al*., 2015; Kim *et al*., 2017; Han *et al*., 2018). Nowadays, it is identified that mitochondrial dysfunction is one of the most prominent features of cancer cells.

Mitochondrion is the major source of cellular reactive oxygen species (ROS). In some cases, the suppression of respiratory activity clearly leads to enhanced generation of ROS (Gottlieb & Tomlinson, 2005). By acting both as mutagens and cellular mitogens (Watson *et al*., 2006; Haruma *et al*., 2018), excessive ROS may change the cellular redox status, induce oxidative stress (Hornsveld & Dansen, 2016) and alter the activities of redox sensitive transcription factors, thus changing gene expression and stimulating cancer cell proliferation (Kumar *et al*., 2008; Schroder, 2019). Therefore, ROS elevation caused by mitochondrial dysfunction may be involved in carcinogenesis.

In this study, we studied the function and molecular mechanism of gastrin in regulating proliferation of gastric cancer cells. We found that the overexpression of gastrin induced mitochondrial dysfunction and inhibited cell apoptosis via promoting mitochondrial ROS production. We further explored the underlying mechanism of gastrin regulating mitochondrial function.

MATERIAL AND METHOD

Cell lines. The human gastric cancer cell line SGC7901 (gifts from Laboratory of Molecular Oncology, Peking University Cancer Hospital) were maintained in DMEM medium (Gibco, Grand Island, NY, USA) supplemented with 5 % fetal bovine serum (FBS, Grand Island, NY, USA), penicillin (100 μg/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5 % CO_2.

Construction of plasmid and transfection. Gastrin cDNA from SGC7901 cells was amplified by RT-PCR and cloned into the *Xho1/BamH1* site of the pcDNA3.1/ myc-his(B-) vector (Invitrogen, Carlsbad, CA, USA) to construct gastrin expression plasmid. Cells were cultured to 60–70 % confluence in 35 mm dishes and then were transfected with gastrin expression plasmid using Lipofectamine2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Selective medium containing 400 g/ml G418 was used to screen stable cell clones overexpressing gastrin 48 h after transfection. SGC7901 cell transfected with gastrin expression plasmid was named as pc-7901-G; and SGC7901 cell transfected with pcDNA3.1/ myc-his(B-) vector was named as pc-7901-vector.

Real-time quantitative PCR. Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, 5 μg of total RNA was reverse transcribed into cDNA with SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed using ABI PRISM7700 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). The primer pairs: gastrin, 5'-GAC GAG ATG CAG CGA CTA TGT-3'(sense) and 5'-GGG TCT GCC ACG AGG TGT-3'(antisense); and β-actin, 5'-CGG GAA ATC GT GCG TGA CAT T-3'(sense) and 5'-CTA GAA GCA TTT GCG GTG GAC-3'(antisense). PCR reaction was conducted in triplicate. β-actin was used as an internal control. The cyclic reaction program was 94 °C denaturation for 30 s, 62 °C annealing for 30 s, 72 °C elongation for 30 s, 40 Cycles, and full elongation at 72 °C for 10min. The mRNA relative expression levels of gastrin were calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunofluorescence analysis of *in situ* protein expression. The cells cultured on slides were fixed with 4 % formaldehyde for 10 min, and permeabilized with 0.5 % Triton X-100 PBS for 15 min at room temperature. The slides were then incubated with primary antibodies: anti-gastrin (1:250, Abcam, Cambridge, MA, USA), anti-CoxIV (1:100, Proteintech, Chicago, USA) and anti-Annexin A2 (1:100, Proteintech, Chicago, USA) overnight at 4 °C. After washing, FITC/TRIRF conjugated secondary antibody (1:50, Zhongshan Jinqiao Biotechnology, Beijing, China) was added and incubated for 1h at 37 °C. DAPI was used to stain the nuclei. The slides were covered with glass cover slips and fluorescence photomicrographs were obtained by confocal laser-scanning microscope (TCS- SP5, Leica, Mannheim, Germany).

MTT assay. The transfected cells were cultured in 96-well plates at a density of 5×10^3 cells/well and 100 μl MTT (5 mg/ml) was added at 24, 48, 72 and 96 h respectively. After 4 h of incubation at 37 °C, the medium was replaced with 200 μl DMSO (Sigma-Aldrich, St Louis, USA) and vortexed to solubilize the formazan for 15 min at room temperature. Optical density (OD) was read at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Three independent experiments were performed.

Soft agar colony formation assay. The colony formation ability of transfected cells in soft agar was determined according to standard protocol. In brief, 5×10^3 cells were trypsinized to single-cell suspension and placed in triplicate into 60 mm dishes in DMEM containing 0.3 % agarose overlying a 0.6 % agarose under-layer. Plates were incubated at 37 °C, 5 % CO_2 for about 28 days and the colonies were examined by fluorescence microscopy and photographed.

Animals. Forty six-week-old female BALB/c nude mice were purchased from Charles River Laboratories (Beijing, China) and housed under specific pathogen free conditions. This study was conducted in strict accordance with the recommendations of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The research protocol with animal experimentation was approved by the ethical review board of Peking University Cancer Hospital (NO. SL-2017010).

Tumorigenicity assay in nude mice. Single-cell suspensions (pc-7901-G cells and pc-7901-vector cells) (5×10^5 in 0.1 ml Hank's buffered salt solution) were subcutaneously injected into the right dorsal flank of nude mice. The tumors were measured every 3 days with a caliper, and the diameters were recorded. Tumor volumes were calculated using the formula: $a^2b/2$ (a represents the length and b represents the width of the tumor). Animal fluorescence imaging was performed using Ami small animal imaging system (Spectral Instruments Imaging Co.)

Western blot. The transfected cells or the cells pretreated with NAC (15 μmol/L) (the inhibitor of ROS; Beyotime, Shanghai, China) or BAY (10 μmol/L) (the inhibitor of NF-κB; Beyotime, Shanghai, China) for 2 h were collected and lysed in lysis buffer. Equal amounts of protein (50 μg) were separated by 12 % SDS–PAGE and transferred to PVDF membrane. The membrane was blocked with 5 % nonfat milk in TBST for 1 h. PVDF membranes were then incubated with the primary antibodies: anti-gastrin (Abcam, Cambridge, MA, USA) (1:800), anti-Cox17 (Abcam, Cambridge, MA, USA) (1:500), anti-Cox5B (Abcam, Cambridge, MA, USA) (1:1000), anti-ATP5J (Proteintech, Chicago, USA)(1:500), anti-NF-kB (Santa Cruz, CA, USA) (1:500), anti-IκB-α (Santa Cruz, CA, USA) (1:500), anti-p-IκB-α (Santa Cruz, CA, USA) (1:500), anti-Bcl-2(US Biological, Boston, USA) (1: 500), anti-Bax (Epitomics, Burlingame, CA, USA) (1:500), anti-Annexin A2 (Abcam, Cambridge, MA, USA) (1:400) and β-actin (Santa Cruz, CA, USA) (1:20000) overnight at 4 °C, and sequentially incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA) (1:2000) for 1 h. The blots were visualized using the ECL system (GE Health Care, Buckinghamshire, UK). Equal loading was confirmed by β-actin detection.

Mitochondrial membrane potential analysis. JC-1 was used to demonstrate the change in the mitochondrial membrane potential ($\Delta\psi m$) of gastric cancer cells. Briefly, cells were seeded in 60 mm plates and on cover slips in 30 mm diameter plates, respectively. After 24 h, cells were washed once with PBS, incubated with 10 μM JC-1 for 2 h at 37 °C, and then washed three times with PBS. The cells on the cover slips were observed for green monomers and red J-aggregates under confocal laser-scanning microscope. The cells in 60 mm plates were collected and analyzed by flow cytometry.

Measurement of ROS production. The production of ROS was monitored by confocal laser-scanning microscope and flow cytometry using DCFH-DA molecular fluorescence probes. Cells were seeded in 60 mm plates and on cover slips in 30 mm diameter plates. DCFH-DA (10 μM) was added and incubated for 30 min at 37 °C in the dark, then washed three times with PBS. The cells on the cover slips were observed for green fluorescence under confocal laser-scanning microscope. The cells in 60 mm plates were collected for flow cytometry analysis.

Isolation of mitochondria. Isolation of mitochondria from gastric cancer cells was performed using mitochondrion isolation kit (Beyotime, Biotechnology, Shanghai, China) according to the manufacturer's protocol. Briefly, 10×10^7 cells were harvested and washed with ice-cold PBS. Cells were incubated with 1 ml of mitochondrion separation liquid for 15 min on ice, and then homogenized with a glass homogenizer for 2 min. The homogenates were centrifuged at 600 g for 10 min and then the supernatants were further centrifuged at 11,000 g for 10 min at 4 ºC. The cytosolic supernatants were decanted and the pellets were resuspended in 50 μl mitochondrial stock solution.

Flow cytometry analysis of cell apoptosis. To detect the apoptosis of cells, Annexin V-FITC apoptosis detection kit (Beijing Baosai Biotech, Beijing, China) was used. Briefly, cells were collected and suspended in pre-chilled PBS. After washing with PBS for 3 times, cells were re-suspended in 200 μl binding buffer with 10 μl Annexin V-FITC and incubated in dark for 15 min at room temperature. Later, 300 μl binding buffer with 5 μl PI solution were added into the cell suspension. Then the apoptotic rate of gastric cancer cells was detected by flow cytometer (BD FACSAria, San Jose, CA, USA) within an hour. Cells in early stages of apoptosis were Annexin V positive and PI negative, whereas cells in the late stages of apoptosis were both annexin V and PI positive.

Co-immunoprecipitation (Co-IP) and protein identification. Cells were lysed by adding 200 uL 85 °C

1×SDS lysate containing 2 uL PMSF to the culture dish (100 mm in diameter). The cell lysates were collected and centrifuged at 12000 rpm for 20 min at 4 °C. The supernatant was taken and quantified. Co-IP assays were performed according to the manufacturer's instructions. After overnight incubation of the lysates with the antibody against gastrin, the immune complex was captured by protein A/G immobilized on agarose beads (Pierce Biotechnology, Rockford, IL), and fractionated by 12 % SDS-PAGE. To identify the unknown proteins, the gel was stained using the sensitive Colloidal Coomassie universal protein dye (Invitrogen, Carlsbad, CA, USA). The band for the protein of interest was excised from the gel and sent to the Taplin Biological Mass Spectrometry Facility for protein identification by mass spectrometry.

iTRAQ and bioinformatics analysis. The pc-7901-G, and the pc-7901-vector cells were lysed in lysis buffer (50 mmol/L Tris–Cl (pH 6.8), 100 mmol/L DTT, 2 % SDS, 10 % glycerol). Equal amounts of protein (50mg) were sent to Beijing Huada Protein Research & Development center for iTRAQ (isobaric tags for relative and absolute quantitation) analysis. The differentially expressed proteins were identified by iTRAQ. The corresponding genes that code these differentially expressed proteins were subjected to GO and KEGG analysis by taking advantage R software and IPA (Ingenuity pathways analysis).

Statistical analysis. Data was analyzed by using statistical software SPSS19.0 (IBM, Armonk, NY, USA). Chi-square test was used for counting data comparison. Differences between two groups were evaluated using two-sided unpaired Student's t-test. Comparison of more than two groups was performed with one-way ANOVA followed by Tukey multiple comparison test. Differences were considered statistically significant at * $p<0.05$ and ** $p<0.01$.

Table I. Diseases and disorders.

Name	p-value	Molecule
Infectious Disease	1.04E-05 - 4.21E-02	31
Cancer	1.82E-04 - 4.97E-02	60
Respiratory Disease	6.45E-04 - 2.83E-02	10
Renal and Urological Disease	8.05E-04 - 3.67E-02	23
Reproductive System Disease	1.98E-03 - 4.95E-02	20

Table II. Molecular and cellular function.

Name	p-value	Molecule
RNAPost-Transcriptional Modification	8.74E-06 - 4.62E-02	11
Cell Signaling	1.94E-05 - 4.90E-02	12
Cellular Growth and Proliferation	1.97E-05 - 4.90E-02	44
Lipid Metabolism	7.47E-05 - 4.90E-02	10
Molecular Transport	7.47E-05 - 4.90E-02	17

RESULTS

Gastrin overexpression promotes gastric cancer cell proliferation in vitro and *in vivo*. To investigate the function of over-expressed gastrin in gastric cancer cells, we constructed pc-7901-G cell model using SGC7901 cell that is characterized by a low endogenous expression level of gastrin and studied the effects of gastrin overexpression on cell proliferation (Fig. 1A and B). The gastrin overexpressing cell clone (pc-7901-G) showed a higher propensity to form large and more macroscopically visible colonies in soft agar than controls (pc-7901-vector) (Fig. 1C and 1D, $p < 0.001$). In addition, using MTT assays we found that pc-7901-G had higher rates of proliferation compared with control cells (Fig. 1E). Subsequently, we studied the effects of gastrin overexpression on cell growth in vivo and the results showed that cell clones (pc-7901-G cell) with gastrin overexpression formed substantially larger tumors in nude mice than the control group (pc-7901-vector) (Fig. 1F).

Identification of the differential expression profiles of proteins in gastrin-overexpressing cell. To identify the molecular pathways underlying the observed effects of gastrin on cell proliferation, we analyzed differentially expressed proteins in gastrin over-expressing cells ((pc-7901-G cell) compared with control cells (pc-7901-vector) by performing iTRAQ analysis. A total of 173 proteins were identified in pc-7901-G cell, with 86 proteins up-regulated and 87 proteins down-regulated. Analysis of the biological functions regulated by these differential proteins using IPA software revealed that in the disease prediction model, the most involved molecule was “tumor”, with a total of 60 molecules involved, accounting for 42 % of the entire model (Table I). In the molecular and cellular function model, the most molecule was related to“cell growth and proliferation”, with 44 molecules involved, accounting for 47 % of the entire model (Table II). At the same time, we analyzed the signaling pathways in which the differential proteins may be involved and found that these proteins were involved in important pathways such as mitochondrial dysfunction, cellular junctions, cell proliferation, cycle regulation, PKA, Wnt signaling pathways (Fig. 2).

The overexpression of gastrin is related to the mitochondrion dysfunction in gastric cancer cells. Among the differentially expressed proteins, we were particularly interested in three proteins, including Cox17, Cox5B and ATP5J, since they were all assembled on mitochondrial oxidation respiratory chain and down-regulated simultaneously. Western blot was used to

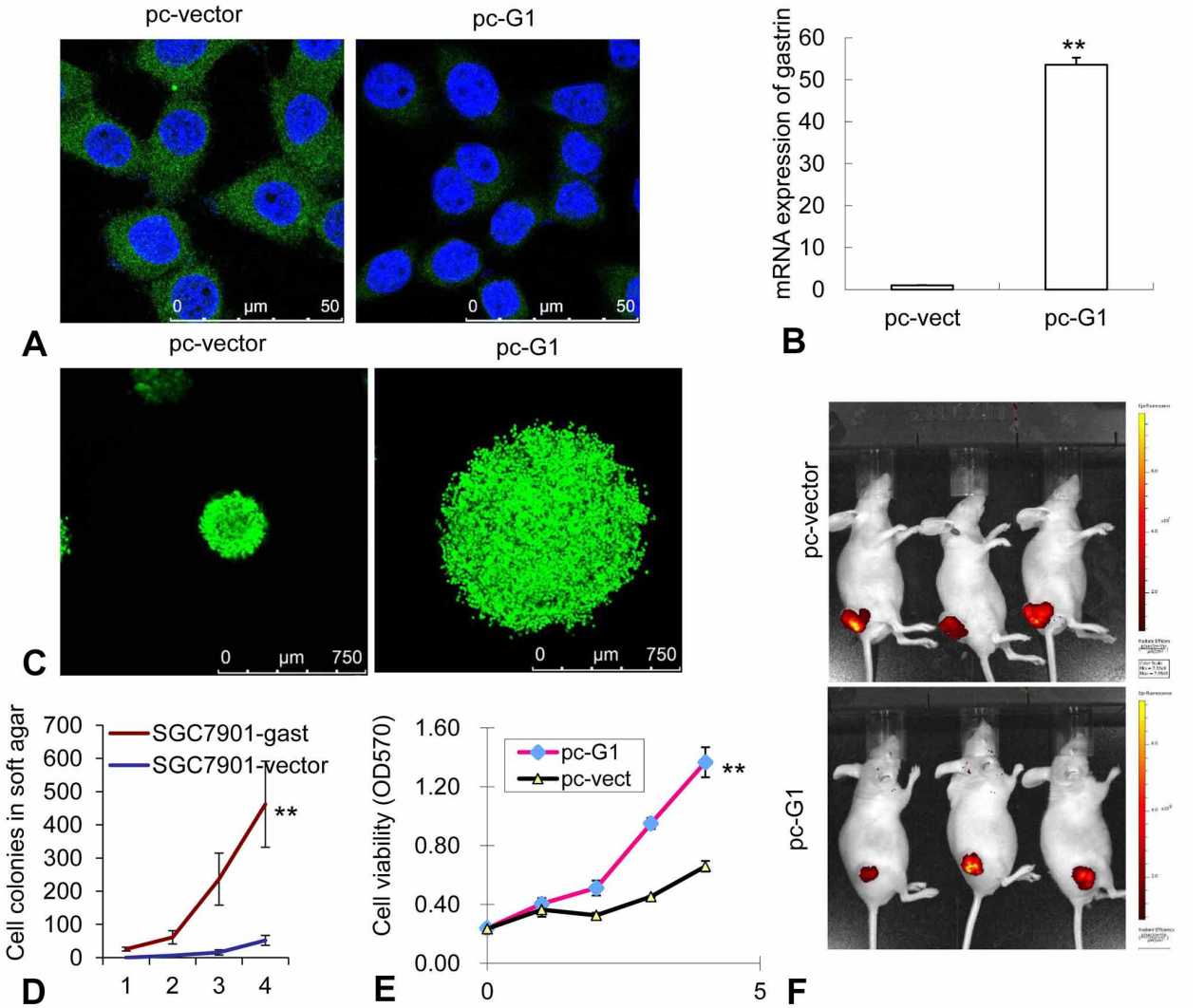

Fig. 1. Overexpression of gastrin in GC cells promotes cell growth. A and B. Ectopic expression of Gastrin in SGC7901 cells detected by immunofluorescence and RT-PCR. C. Representative image of colony formation by overexpressed cells. D. The numbers represent the mean number of colonies of three independent experiments. E. MTT assay detected growth of SGC7901 cells after increase gastrin expression. Bars represent standard deviations (SD) of three independent experiments performed in triplicate. F. Animal fluorescence imaging of tumor-bearing mice model. (**p < 0.01 compared with control).

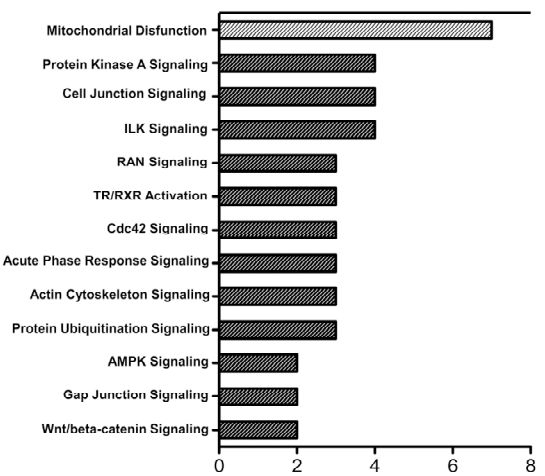

Fig. 2. Bioinformatics analysis of differentially expressed proteins in gastrin-overexpression cells. Signal pathway analysis of the differentially expressed proteins.

confirm iTRAQ results. As shown in Figure 3A, Cox17, Cox5B and ATP5J were under expressed in gastrin overexpression cell (SGC7901-G1) compared with control cell (SGC7901-vect). These results indicate that gastrin may induce mitochondrial dysfunction via down-regulating Cox17, Cox5B and ATP5J.

Mitochondrial membrane potential is a direct indicator of response function. Furthermore, we used JC-1 staining to detect mitochondrial membrane potential in gastrin overexpressing cells by flow cytometry and laser-scanning confocal microscopy. As shown in Figure 3B, the flow cytometry results showed that $\Delta\psi\mu$ was significantly decreased in SGC7901 cells with gastrin overexpression. The results of laser-scanning confocal microscopy were consistent with flow cytometry results (Fig. 3C). Our results indicate that the overexpression of gastrin leads to the decline of mitochondrial membrane potential in gastric cancer cells, suggesting gastrin overexpression may lead to mitochondrial dysfunction.

Gastrin induces ROS generation and NF-κB activation and affects Bax and *Bcl-2* expression. Mitochondria are the major source of ROS and our results suggest that overexpression of gastrin in gastric cancer cells led to mitochondrial dysfunction. Therefore, we speculate that gastrin overexpression may upregulate ROS level via inducing mitochondrial dysfunction, and we studied the ROS generation by DCFH-DA staining in the transfected model cells. Laser-scanning confocal microscopy (Fig. 4A, top) and flow cytometry (Fig. 4A, bottom) analyses revealed that high levels of gastrin led to significantly increase of ROS generation.

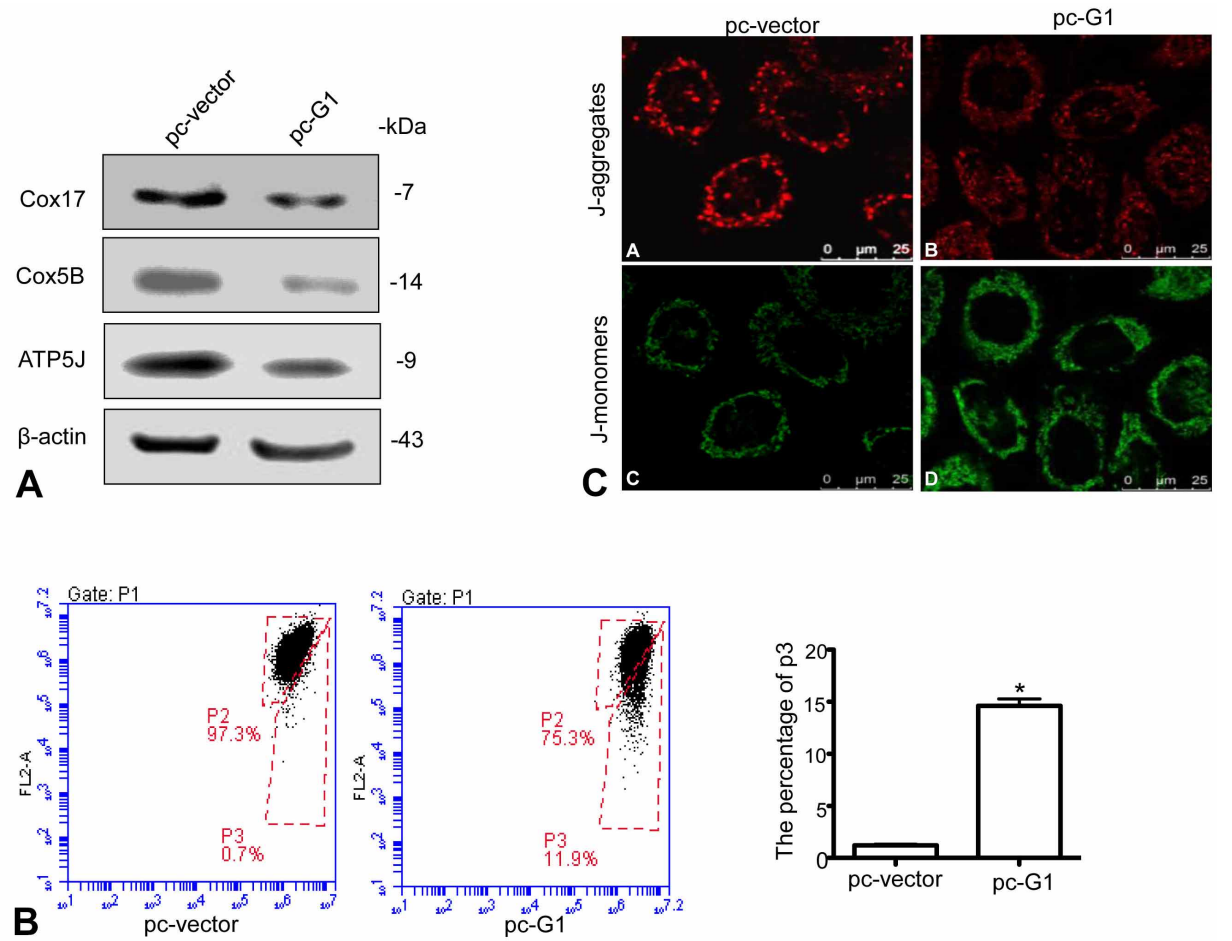

Fig. 3. Overexpression of gastrin leads to the decline of mitochondrial membrane potential in GC cells. A. Western blot analysis verified iTRAQ results. Representative Western blots of differently expressed proteins identified by iTRAQ in the gastrin overexpressed cells. B. Dym analyzed by flow cytometry. Cell populations with higher and lower JC-1 aggregated staining are marked with high percentage of p3 and low percentage of p3, respectively. Each column represents the mean ±SD of three independent experiments. Compared with pc-vector, *P<0.05. C. Mitochondrial membrane potential (Dym) evaluated by JC-1 staining. Representative fluorescence micrographs of JC-1-stained overexpressed cells were shown. Note the accumulation of red J-aggregates in control cells (images b) and accumulation of green J-monomers in overexpressed cells (images d).

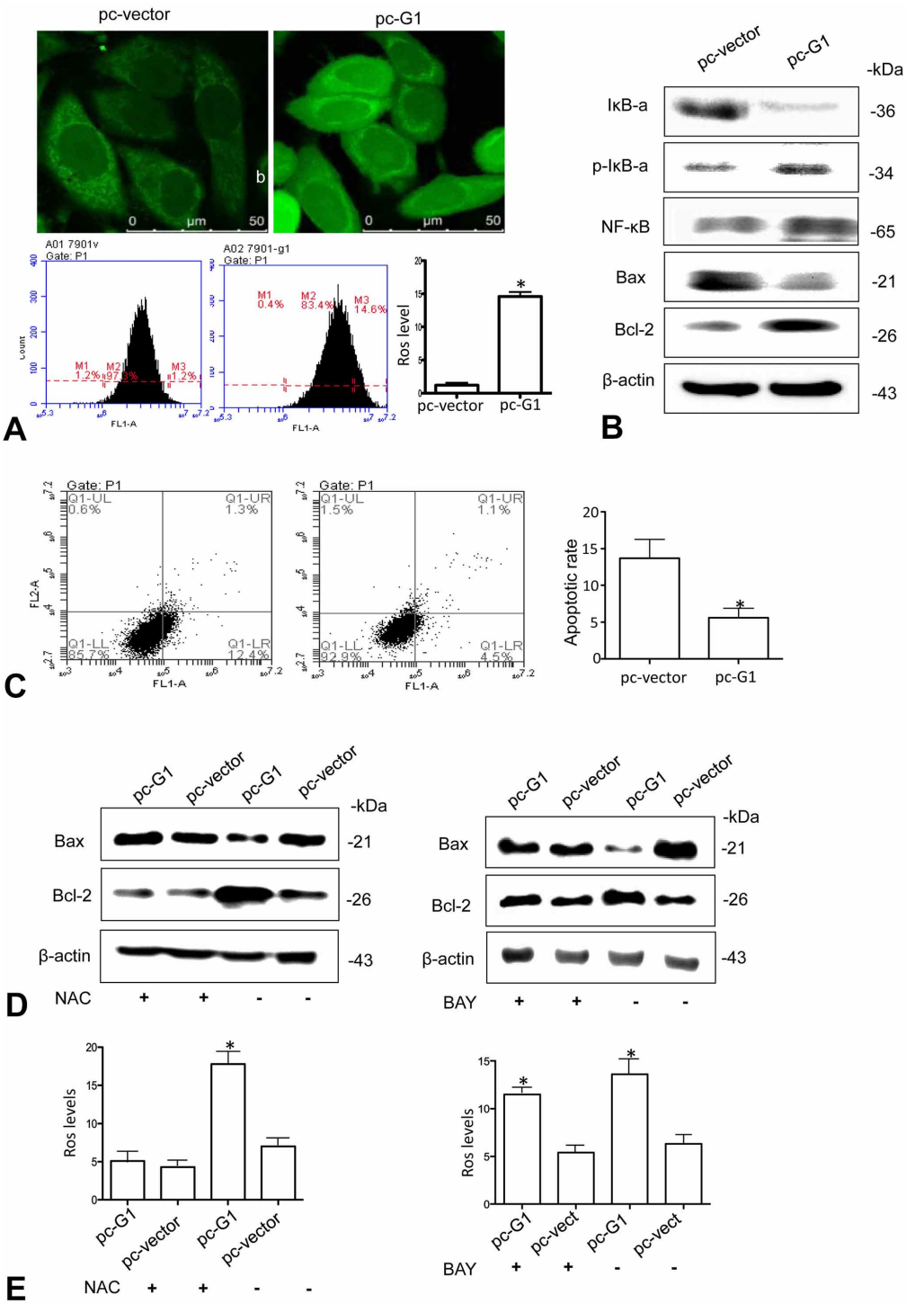

Fig. 4. Gastrin induces ROS generation, NF-κB activation and affects Bax and Bcl-2 expression. A. DCFH-DA staining detected cell ROS production. Top: DCFH-DA staining analyzed by a laser-scanning confocal microscopy (magnification, 100×). Bottom: Flow cytometry analysis detected ROS using DCFH-DA molecular probe. Data are expressed as means ± SD (*$p < 0.05$ compared with controls). B. Western blot detection of protein expression levels of IκB-α, p-IkB-α, NF-κB, Bcl-2 and Bax in gastrin-overexpression cells. β-actin was used as a loading control. C. Flow cytometry assays showed the apoptosis of gastrin-overexpression cells. Data are expressed as means ± SD (*$p < 0.05$ compared with pc-vector). D. Western blot detection of protein expression levels of Bcl-2 and Bax after treatment with NAC (left panel) and BAY (right panel). E. Flow cytometry analysis detected ROS using DCFH-DA molecular probe after treatment with NAC (left panel) and BAY (right panel). Data are expressed as means ± SD (*$p < 0.05$ compared with controls).

Given that ROS can activate expression of redox-sensitive transcription factor (Fiorentino *et al*., 2013), we next determined the effect of gastrin-induced ROS on the expression of some transcription factor. Western blot analysis showed that overexpression of gastrin in SGC7901 cells led to down-regulation of IκB-α expression accompanied with up-regulation of p-IκB-α and NF-κB expression (Fig. 4B). These data suggest that gastrin can promote NF-κB activity.

Since both Bax and Bcl-2 proteins are regulated at the posttranscriptional level by NF-κB (Visvader, 2011; Venditti *et al*., 2013), Western blot was used to study the expression level of Bax and Bcl-2 in gastrin overexpressing SGC7901 cells, and the results showed that gastrin overexpression induced the reduction of Bax and elevation of Bcl-2 (Fig. 4B). Furthermore, we detected the apoptosis rate of the constructed cell models to study the effect of gastrin on GC cell apoptosis (Fig. 4C). The results showed that the apoptosis rate was higher in GC cell with gastrin knockdown, suggesting gastrin inhibit apoptosis of GC cell. These results suggest that gastrin overexpression may up-regulate Bcl-2 and down-regulate Bax via promoting NF-κB activity to inhibit GC cell apoptosis.

To further identify the underlying mechanism of gastrin inducing expression level change of Bcl-2 and Bax, we pretreated cell models with the antioxidant NAC, a ROS scavenger. Pretreatment with NAC eliminated the effect of gastrin on Bax and Bcl-2 expression in gastrin overexpressing cells (Fig. 4D), and induced a significant reduction in ROS (Fig. 4E), indicating that gastrin induced expression changes of Bax and Bcl-2 is ROS-dependent. Besides, cell models were treated with BAY 11-7082, an inhibitor of NF-κB, and the effect of gastrin on Bax and Bcl-2 expression were eliminated but the ROS production was not affected (Fig. 4D and 4E), suggesting that increased activity of NF-κB is down-stream of ROS signaling and up-stream of Bax and Bcl-2 in gastrin induced apoptosis pathways.

Co-IP analysis and co-localization of mitochondrial proteins reveals a novel interaction between gastrin and Annexin A2. Co-IP assay was performed to determine whether gastrin can interact with mitochondrial proteins. Gel electrophoresis of co-immunoprecipitates prepared using the anti-gastrin antibody consistently showed six bands of additional proteins compared with the immunoprecipitates from control IgG (data not shown). Mass spectra analysis of the excised protein bands identified Annexin A2 in the gastrin co-immunoprecipitates (Fig. 5A).

To determine whether gastrin and Annexin A2 are located in the mitochondria, confocal immunofluorescent analysis was performed. Results in Figure 5B showed that there were significant amounts of gastrin proteins that overlapped with the mitochondrial marker, CoxIV. Similarly, CoxIV showed significant co-localization with the Annexin A2. Furthermore, we have also fractionated mitochondrial proteins from the cancer cells and performed Western blot analysis of the fractionated proteins. There were significant levels of gastrin and Annexin A2 protein in the mitochondrial fraction as shown in Figure 5C.

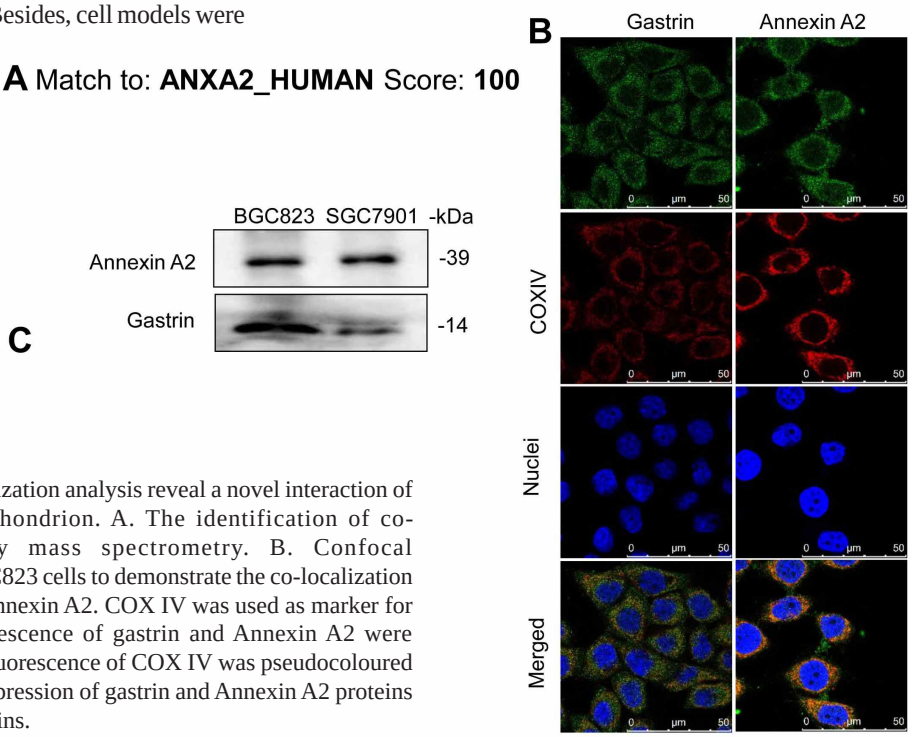

Fig. 5. Mass spectrometry and co-localization analysis reveal a novel interaction of gastrin with Annexin A2 on mitochondrion. A. The identification of co-immunoprecipitating proteins by mass spectrometry. B. Confocal immunofluorescence of wild-type BGC823 cells to demonstrate the co-localization of gastrin and mitochondrial protein Annexin A2. COX IV was used as marker for intracellular mitochondria. The fluorescence of gastrin and Annexin A2 were pseudocolored as green, whereas the fluorescence of COX IV was pseudocoloured as red. C. Western blot analyzed the expression of gastrin and Annexin A2 proteins in the fractionated mitochondrial proteins.

DISCUSSION

Gastrin can regulate acid secretion and proliferation of gastric epithelium. However, the molecular pathways by which gastrin regulates tumor cell growth remain unknown. In this study, by inducing overexpression of gastrin in gastric cancer cell line SGC7901, we found an accelerated cellular growth rate, an enhanced ability of colony formation as well as tumor growth *in vivo*. These findings suggest that gastrin is involved in gastric cancer cell proliferation.

In order to better understand the molecular mechanism of biological phenomena, we established the differential protein expression spectrum before and after increasing gastrin expression using ITRAQ analysis. Totally, 173 differentially expressed proteins were identified and it was found that the most involved disease model was "tumor". In the molecular and cellular function model, the most differential proteins were involved in the function of "cell growth and proliferation". This result clarified the key role of gastrin in the development of gastric cancer from a proteomic point of view and further confirmed that gastrin had an important regulatory role in the growth and proliferation of tumor cells.

In the subsequent signal pathway analysis, the effect of gastrin on mitochondrial oxidative respiration function attracted our attention. Classical biological pathway analysis showed that gastrin had a significant effect on the mitochondrial function. The three proteins on the mitochondrial oxidative respiratory chain complex were all down-regulated after increasing gastrin expression. This result was also confirmed by Western blot. So far, there have been no relevant reports about the influence of gastrin on mitochondrial protein expression. However, dysregulation of mitochondrial respiratory chain-related protein expression in tumors has become an increasing concern. For example, depressed expression levels for all MRC (mitochondrial respiratory chain) complexes have been be detected in breast-infiltrating ductal carcinoma cells (Putignani *et al*., 2008, 2012), particularly for NDUFS3 (complex I), 2 UQCRC2 (complex III), COX 1 (complex IV) and ATPà (complex V). Similarly, Wallace *et al*. (2016) detected altered expression of ND6 1, COI, and ATP6 (complex V) in colorectal cancer. A proteomic analysis revealed altered expression of several MRC proteins of MCF-7 cells treated with benzo[a]pyrene (Hooven & Baird, 2008).

Mitochondria are considered as the main source of ROS (Venditti *et al*., 2013). It is reported that the defects of ETC (blockade of complex I, oxidation of complex I or II substrates) can lead to remarkably increase mitochondrial ROS production (Mazure & Pouyssegur, 2010). Our data demonstrated that gastrin overexpressing cells harbored dysfunctional mitochondria with an increase of ROS production, suggesting that gastrin up-regulation is a critical event that induces mitochondria derived ROS elevation. Compelling evidence suggest that the increased ROS stress in cancer cells has a pivotal role in the acquisition of the hallmarks of cancer (Hanahan & Weinberg, 2011), including disruption of cell death signaling (Clerkin *et al*., 2008). Meanwhile, NF-κB is a redox-sensitive transcription factor that is activated by increased levels of ROS (Pelicci *et al*., 2013) and it also has been shown to induces the expression of some members of the anti-apoptotic proteins (Renault *et al*., 2017).

Since our results showed that elevation of gastrin expression inhibited apoptosis, we tested whether gastrin-induced ROS modified anti-apoptosis signaling. Our results further showed that gastrin induced an increase in the expression of anti-apoptotic Bcl-2 and a decrease in pro-apoptotic protein Bax. Furthermore, scavenged ROS production completely inhibited the gastrin-induced increase of Bcl-2 and rescued the decrease of Bax. These data demonstrated that gastrin-mediated regulation of Bcl-2 and Bax occurs in a ROS-dependent manner.

Since Bax and Bcl-2 are reported to be regulated at the posttranscriptional level by NF-κB (Clerkin *et al*., 2008; Putignani *et al*., 2008, 2012), we further investigated the effect of gastrin on the NF-κB. Our results showed that gastrin up-regulated NF-κB activation through degradation of IκB-α, and that inhibiting the activity of NF-κB also abolished gastrin-induced up-regulation of Bcl-2 and down-regulated of Bax but did not influence ROS production, suggesting that NF-κB activation is upstream of Bax and Bcl-2 and downstream of ROS in gastrin mediated pathway. Taken together, these results suggest that gastrin plays a central role in anti-apoptosis of gastric cancer cells via up-regulating ROS production to activate NF-κB and further leading to Bax/Bcl-2 ratio decrease.

In addition, the mechanism of gastrin leading to mitochondria dysfunction is unclear. The Casein kinase I epsilon has been shown to interact with mitochondrial protein ANT2 and promote the growth of ovarian cancer cells (Rodriguez *et al*., 2012). So, we assume that the gastrin may bind to mitochondria to regulate mitochondrial function. Through Co-IP and spectrometry, we found that Annexin A2 interacted with gastrin in mitochondria. It has been reported that Annexin A2 can form high-molecular weight complexes with prohibitin in the mitochondria (Bacher *et al*., 2002). More specifically, Annexin A2 is reported as a novel gastrin receptor in colon cancer (Singh, 2007). These

observations together suggest that gastrin may anchor in mitochondria membrane through binding to Annexin A2. The assumption was validated by confocal immunofluorescence co-localization experiments and Western blot analysis with the mitochondrial fraction.

In conclusion, our research reveals that gastrin harbors tumorigenic property and is critical to cellular proliferation of gastric cancer cells. It can interact with outer mitochondria membrane protein Annexin A2, affect mitochondrial function, and promote production of mitochondrial derived ROS, which leads to activation of redox-sensitive transcription factor NF-κB and increases anti-apoptosis associated proteins. These newly identified mechanisms of gastrin in gastric cancer cells indicate the potential role of mitochondria and ROS in gastrin-dependent gastric cancer progression and may provide new targets for developing antioxidant-based anticancer therapy.

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LIU, Y.; ZHU, J.; LIU, J.; SU, Z.; MA, X.; WANG, H.; XING, Y.; ZHAO, J. La sobreexpresión de gastrina inhibe la apoptosis de las células de cáncer gástrico a través de especies reactivas de oxígeno y disfunción mitocondrial mediada por anexina A2. *Int. J. Morphol., 41(1)*:308-318, 2023.

RESUMEN: La gastrina juega un papel vital en el desarrollo y progresión del cáncer gástrico (CG). Su expresión está regulada al alza en tejidos de CG y en varias líneas celulares de CG. Sin embargo, el mecanismo subyacente aun no se ha investigado. El objetivo de este estudio fue investigar el papel y el mecanismo de la gastrina en la proliferación de CG. El modelo de células CG que sobre expresan gastrina se construyó usando células SGC7901. Luego, las proteínas expresadas diferencialmente se identificaron mediante análisis iTRAQ. A continuación, utilizamos la citometría de flujo y la inmunofluorescencia para estudiar el efecto de la gastrina en el potencial mitocondrial y la producción de ROS derivada de las mitocondrias. Finalmente, estudiamos el mecanismo subyacente de la gastrina que regula la función mitocondrial utilizando Co-IP, espectrometría de masas e inmunofluorescencia. La sobreexpresión de gastrina promovió la proliferación de células CG *in vitro* e *in vivo*. Un total de 173 proteínas se expresaron de manera diferente entre los controles y las células con sobreexpresión de gastrina y la mayoría de estas proteínas estaban implicadas en la tumorigenesis y la proliferación celular. Entre estas, Cox17, Cox5B y ATP5J, todas localizadas en la cadena respiratoria mitocondrial, estaban reguladas a la baja en las células con sobreexpresión de gastrina. Además, la sobreexpresión de gastrina provocó una disminución del potencial mitocondrial y un aumento de las ROS derivadas de las mitocondrias. Por otra parte, la generación de ROS inducida por gastrina resultó en la inhibición de la apoptosis celular mediante la activación de NF-κB, inhibiendo la expresión de Bax y promoviendo la expresión de Bcl-2. Finalmente, encontramos que la gastrina interactuaba con la proteína de membrana mitocondrial Anexina A2 usando Co-IP y espectrometría de masas. La sobreexpresión de gastrina inhibe la apoptosis de las células CG al inducir la disfunción mitocondrial a través de la interacción con la proteína mitocondrial Anexina A2, luego regula el aumento de la producción de ROS para activar NF-κB y conduce aún más a la disminución de la relación Bax/Bcl-2.

PALABRAS CLAVE: Gastrina; Cáncer gástrico; Mitocondrias; ROS; NF-kB; Anexina A2

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