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ORIGINAL ARTICLE

Basic Study

MiR-204-3p overexpression inhibits gastric carcinoma cell proliferation by inhibiting the MAPK pathway and RIP1/MLK1 necroptosis pathway to promote apoptosis

Xia Li, Joanna J Tibenda, Yi Nan, Shi-Cong Huang, Na Ning, Guo-Qing Chen, Yu-Hua Du, Ya-Ting Yang, Fan-Di Meng, Ling Yuan

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Abstract

BACKGROUND

Gastric carcinoma (GC) is the third most frequent cause of cancer-related death, highlighting the pressing need for novel clinical treatment options. In this regard, microRNAs (miRNAs) have emerged as a promising therapeutic strategy. Studies have shown that miRNAs can regulate related signaling pathways, acting as tumor suppressors or tumor promoters.

AIM

To explore the effect of miR-204-3p on GC cells.

METHODS

We measured the expression levels of miR-204-3p in GC cells using quantitative real-time polymerase chain reaction, followed by the delivery of miR-204-3p overexpression and miR-204-3p knockdown vectors into GC cells. CCK-8 was used to detect the effect of miR-204-3p on the proliferation of GC cells, and the colony formation ability of GC cells was detected by the clonal formation assay.

The effects of miR-204-3p on GC cell cycle and apoptosis were detected by flow cytometry. The BABL/c nude mouse subcutaneous tumor model using MKN-45 cells was constructed to verify the effect of miR-204-3p on the tumorigenicity of GC cells. Furthermore, the study investigated the effects of miR-204-3p on various proteins related to the MAPK signaling pathway, necroptosis signaling pathway and apoptosis signaling pathway on GC cells using Western blot techniques.

RESULTS

Firstly, we found that the expression of miR-204-3p in GC was low. When treated with the lentivirus overexpression vector, miR-204-3p expression significantly increased, but the lentivirus knockout vector had no significant effect on miR-204-3p. *In vitro* experiments confirmed that miR-204-3p overexpression inhibited GC cell viability, promoted cell apoptosis, blocked the cell cycle, and inhibited colony formation ability. *In vivo* animal experiments confirmed that miR-204-3p overexpression inhibited subcutaneous tumorigenesis ability in BABL/c nude mice. Simultaneously, our results verified that miR-204-3p overexpression can inhibit GC cell proliferation by inhibiting protein expression levels of KRAS and p-ERK1/2 in the MAPK pathway, as well as inhibiting protein expression levels of p-RIP1 and p-MLK1 in the necroptosis pathway to promote the BCL-2/BAX/Caspase-3 apoptosis pathway.

CONCLUSION

MiR-204-3p overexpression inhibited GC cell proliferation by inhibiting the MAPK pathway and necroptosis pathway to promote apoptosis of GC cells. Thus, miR-204-3p may represent a new potential therapeutic target for GC.

Key Words: miR-204-3p; Gastric carcinoma; MAPK signaling pathway; Apoptosis; Necroptosis

Core Tip: Gastric carcinoma (GC) is a global health problem that seriously endangers human life; therefore, it is important to identify effective treatment targets. In this regard, microRNAs (miRNAs) have emerged as a promising therapeutic strategy. Studies have shown that miRNAs regulated signaling pathways, acting as tumor suppressors or tumor promoters. In this study, we first verified the inhibitory effect of miR-204-3p overexpression on GC cells through *in vitro* and *in vivo* experiments. Simultaneously, miR-204-3p overexpression induced GC cell apoptosis by inhibiting the MAPK pathway and the necroptosis pathway. Thus, miR-204-3p may represent a new potential therapeutic target for GC.

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INTRODUCTION

Gastric carcinoma (GC) is a gastrointestinal tumor and the third major cause of cancer-related death[1,2]. Early clinical symptoms are mild or asymptomatic, resulting in difficult diagnosis and a low patient survival rate[3,4]. Currently, the clinical therapy of GC primarily consists of radiotherapy, chemotherapy, and surgical excision, but the therapeutic effect is unsatisfactory[5,6]. Therefore, feasible targeted therapies are particularly important for GC patients. As research has progressed, molecular targets have been found to have a role in the occurrence and development of GC.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by binding to the 3'-untranslated region of target mRNA[7]. With further study in genetic engineering, it has been confirmed that miRNAs regulate different signaling pathways to take part in important cellular processes[8]. miRNAs can act as tumor promoters or inhibitors to target mRNA to regulate GC proliferation, metastasis, angiogenesis, and drug resistance[9-11]. Consequently, it is necessary to strengthen miRNA research for the diagnosis and clinical treatment of GC.

MiR-204-3p has demonstrated efficacy in treating various pathologies, including retinopathy, diabetes, and cancer[12-15]. Crucially, miR-204-3p is underexpressed in melanoma, thyroid carcinoma, glioma and bladder carcinoma, and is related to patient prognosis[16-19]. However, the mechanism of miR-204-3p in GC remains unclear.

Our team's previous research confirmed that the expression of miR-204-3p in GC tissue is low and is associated with poor prognosis in GC patients. In addition, we also verified that KRAS is its direct target[20]. However, the anti-GC effect of miR-204-3p still requires further research to support the possibility of miR-204-3p becoming a new target for the treatment of GC. In this study, we focused on determining the impact of miR-204-3p on GC cells phenotype and its anti-GC molecular mechanism, to provide theoretical support for the treatment of GC by miR-204-3p.

Table 1 Primer sequences

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')
KRAS	TGTGGACGAATATGATCCAACA	GCAAATACACAAAGAAAGCCCT
ERK1	ATGTCATCGGCATCCGAGAC	GGATCTGGTAGAGGAAGTAGCA
ERK2	TACACCAACCTCTCGTACATCG	ATGTCTGAAGCGCAGTAAGATT
GAPDH	CACCCACTCCTCCACCTTTGA	TCTCTCTTCCTCTTGTGCTCTCTTGC
miR-204-3p	CAAGTCGCTGGGAAGGCAA	CAGTGCAGGGTCCGAGGT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

MATERIALS AND METHODS

Cell culture and cell transfection

A normal human gastric epithelial cell line (GES-1) and three GC cell lines (HGC-27, AGS and MKN-45) were acquired from BNCC (Beijing, China). MKN-45 and HGC-27 cells were cultured in RPIM-1640 medium, AGS cells in DMEM/F-12 medium and GES-1 cells in DMEM medium. All culture media were purchased from Gibco (United States), and were supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum. Genechem (Shanghai, China) provided the green fluorescent protein-labeled miR-204-3p overexpression lentiviral vector (OE group), miR-204-3p knockdown lentiviral vector (KD group) and empty lentiviral vector (NC group), which were then transfected into HGC-27, MKN-45 and AGS cells using the tool virus user manual as a guide.

CCK-8 assay

Cell viability was evaluated using the CCK-8 assay. Specifically, lentivirus-transfected AGS and HGC-27 cells were seeded into 96-well plates (5000 cells/well) and cultured for 24 h, 48 h and 72 h, respectively. Following this step, each well was treated with 10 μL CCK-8 reagent (MedChemExpress, United States), and a microplate reader was used to measure the value at 450 nm following incubation for 2 h at 37°C.

Colony formation assay

Lentivirus-transfected AGS and HGC-27 cells were inoculated into 6-well plates (500 cells/well), and fixed with 4% paraformaldehyde for 30 min after 2 wk of continuous culture. Next, the fixing solution was washed off and the cells were stained using 0.5% crystal violet for 10 min. Finally, the cell clones were photographed and statistically analyzed based on clone sizes (diameter > 1 mm).

Flow cytometric analysis

Cell cycle was confirmed using the cell cycle kit (KeyGEN BioTECH, China). Lentivirus-transfected HGC-27 and AGS cells were collected at 1 × 10^6 cells/mL, and then fixed overnight at 37°C with 4% paraformaldehyde. On the second day, the fixing solution was washed off with PBS and 500 μL cell cycle detection working solution (Rnase A:PI = 1:9) was added. The distribution of various groups of cells in the cell cycle was detected after they had reacted for 30 min.

The Annexin V-APC/7-AAD double staining kit (KeyGEN BioTECH, China) was used to confirm apoptosis (early apoptosis and late apoptosis) in each group. Lentivirus-transfected AGS and HGC-27 cells were collected, and 500 μL Binding Buffer, 5 μL Annexin V-APC and 5 μL 7-AAD were added sequentially and gently mixed. Apoptosis was observed after the cells had reacted for 10 min.

Western blot

The expression of related proteins in lentivirus-transfected AGS and HGC-27 cells was detected. Firstly, total protein was extracted with RIPA (Epizyme Biotech, China) from GC cells and their content was confirmed using the BCA assay (Epizyme Biotech, China). Next, the proteins were isolated and transferred onto a polyvinylidene fluoride membrane, which was sealed with 5% skim milk powder, soaked in primary antibody and incubated overnight. On the second day, it was washed with TBST and soaked in HRP-linked secondary antibody (1:1000, 7074/7076, CST, United States) for 1 h. Finally, protein bands were visualized with ECL reagent (KeyGEN BioTECH, China), and the gray values of the protein bands were analyzed using Image J. GAPDH or β-tubulin was used as an internal control to standardize target proteins. p-ERK1/2 (1:5000, 4370), ERK1/2 (1:5000, 9102), RIP1 (1:1000, 3493), p-RIP1 (1:1000, 65746), MLK1 (1:1000, 5029) and p-MLK1 (1:1000, 91689) antibodies were purchased from CST. BAX (1:2000, ab32503), Caspase-3 (1:1000, ab13847), BCL-2 (1:5000, ab182858) and KRAS (1:1000, ab275876) antibodies were purchased from Abcam.

Quantitative real-time polymerase chain reaction

The related genes expressed in each group of GC cells were determined using quantitative real-time polymerase chain reaction (qRT-PCR). Briefly, total RNA was extracted from GC cells using TRIzol reagent (Invitrogen, United States), then cDNA was synthesized (Takara Bio, Japan) and gene expression levels were measured (Takara Bio, Japan). The primer sequences are shown in Table 1. U6 or GAPDH was used as a housekeeping gene, and target genes were calculated using

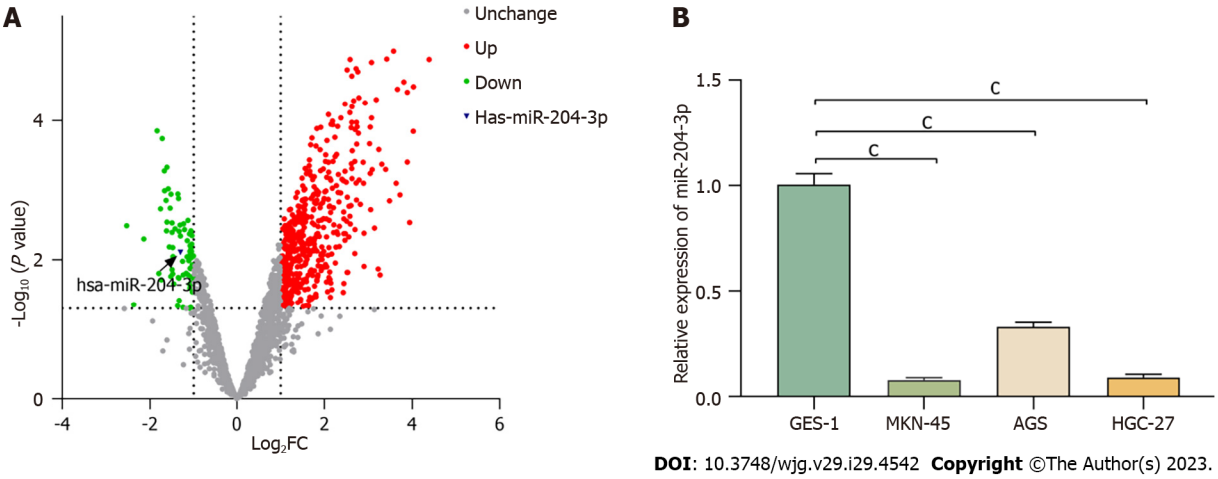

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Figure 1 Expression of miR-204-3p in gastric carcinoma. A: The expression level of miR-204-3p in gastric carcinoma tissues and para-carcinoma tissues was analyzed by the GEO database; B: The expression level of miR-204-3p in normal gastric mucosal epithelial cells and gastric carcinoma cells, $^{c}P < 0.001$.

the $2^{-\Delta\Delta Ct}$ method.

Tumor xenograft experiment

BABL/c nude mice (male, 4 wk, SPF) were provided by the Animal Laboratory Center of Ningxia Medical University. The animal protocol (IACUC-NYLAC-2022-251) was approved by the Institutional Animal Care and Use Committee of Ningxia Medical University. Following 7 d of adaptive feeding in BALB/c nude mice, lentivirus-transfected MKN-45 cells were cultured, and the cell concentration adjusted to 5×10^7 cells/mL. A suspension containing 100 μL cells was slowly subcutaneously injected into the back of nude mice, which were then returned to their cage for feeding. Growth of the subcutaneous tumor and mouse body weight were observed daily. The tumor volume (V) was calculated by $V = (W^2 \times L)/2$ (long diameter: L; short diameter: W). When the tumor on the back grew to an appropriate size and conformed to animal ethics, the animals were killed by CO_2 and photographed.

Differential expression analysis of miRNAs

We used the GEO database (https://www.ncbi.nlm.nih.gov/geo/) to screen differentially expressed miRNAs. We searched the keywords " miRNAs" and "gastric carcinoma, and downloaded Differential expression microarrays of miRNAs (series: GES79973). Then adjusted for $P < 0.05$, $|\log_2 (\text{fold change})| > 1$, and analyzed significant miRNAs.

Statistical analysis

The statistical methods used in this study were reviewed by Li-Qun Wang from the Department of Epidemiology, Department of Medical Statistics, Institute of Public Health and Management, Ningxia Medical University. All data represent the mean ± SD of at least three independent samples. Statistical analysis was conducted using SPSS 27.0 and GraphPad Prism 8.0. One-way analysis of variance was used to analyze the differences between groups. $P < 0.05$ indicated a statistically significant difference.

RESULTS

MiR-204-3p was underexpressed in GC

In our previous study, we analyzed 40 pairs of tissue samples and discovered that miR-204-3p expression was lower in GC tissues compared to normal tissues[20]. To further validate this finding, we utilized the GEO database to identify differentially expressed miRNAs in GC tissues and paracancerous tissues, which confirmed the downregulation of miR-204-3p in GC tissues (Figure 1A). Subsequently, we investigated miR-204-3p expression between GC cell lines (AGS, MKN-45 and HGC-27) and the normal gastric epithelial cell line GES-1. The results revealed that miR-204-3p was underexpressed in MKN-45, AGS, and HGC-27 cells compared to GES-1 cells (Figure 1B).

MiR-204-3p inhibited GC cell viability

Lentivirus transfection caused miR-204-3p overexpression and miR-204-3p knockdown in AGS and HGC-27 cells. The transfection rate was found to be approximately 90% after 72 h, indicating high transfection efficiency (Figure 2A). The qRT-PCR results demonstrated that the miR-204-3p overexpression lentivirus significantly upregulated its expression compared to the NC group in AGS and HGC-27 cells, but the miR-204-3p knockdown lentivirus did not result in downregulation of its expression (Figure 2B and C). These findings indicated that miR-204-3p overexpression in GC cells was highly satisfactory, while miR-204-3p knockdown does not yield meaningful outcomes. Subsequently, the cell viability assay showed that miR-204-3p overexpression significantly inhibited GC cell viability (Figure 2D and E).

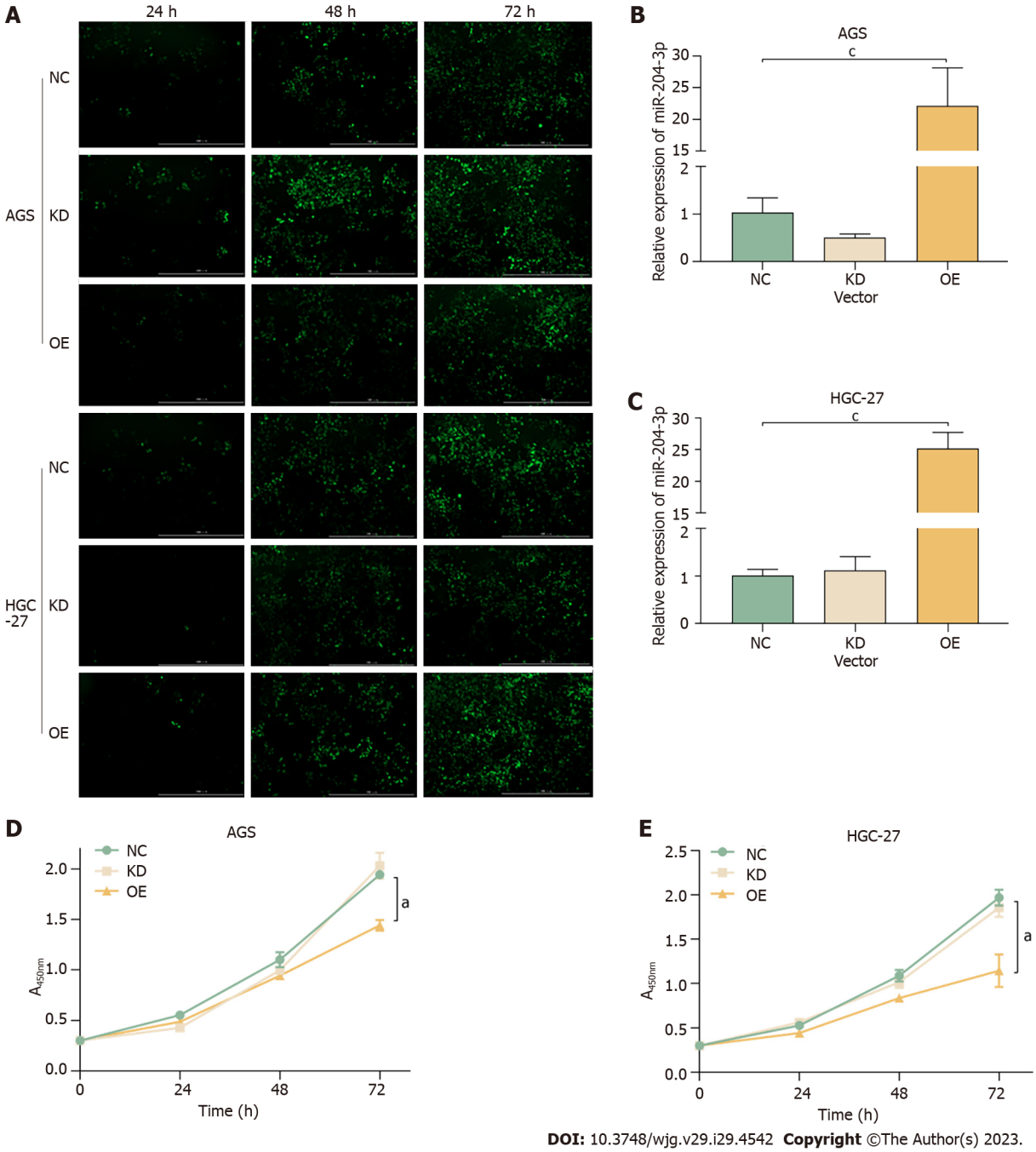

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Figure 2 Effect of miR-204-3p on AGS and HGC-27 cell viability. A: Transfection efficiency of lentiviral vector. Scale bar: 1000 μm, with representative images shown, fluorescence distribution of green fluorescent protein in cells indicating transfection efficiency; B and C: The expression level of miR-204-3p in lentivirus transfected AGS and HGC-27 cells; D and E: Effect of miR-204-3p on AGS and HGC-27 cell viability. $^{a}P < 0.05$, $^{c}P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

MiR-204-3p inhibited the colony forming ability of GC cells

The impact of miR-204-3p on the colony forming ability of GC cells was evaluated. The crystal violet positive staining in the miR-204-3p overexpression group decreased (Figure 3A and B), and simultaneously, the number of clones formed in AGS and HGC-27 cells was distinctly reduced compared to the NC group (Figure 3C and D). This indicated that miR-204-3p overexpression inhibited the colony forming ability of GC cells.

MiR-204-3p promoted GC cell apoptosis

We evaluated apoptosis by collecting cells from each group (Figure 4). The apoptosis rates of AGS cells were 2.53 ± 0.12%, 3.73 ± 0.83% and 10.6 ± 0.70% in the NC, KD and OE groups, respectively (Figure 4A and C). Among HGC-27 cells, the apoptosis rates were 9.47 ± 0.58%, 7.87 ± 1.63% and 18.40 ± 1.27% in the NC, KD and OE groups, respectively (Figure 4B and D). The findings revealed that HGC-27 and AGS cells with miR-204-3p overexpression had a notably higher apoptosis rate, which indicated that miR-204-3p overexpression stimulated GC cell apoptosis.

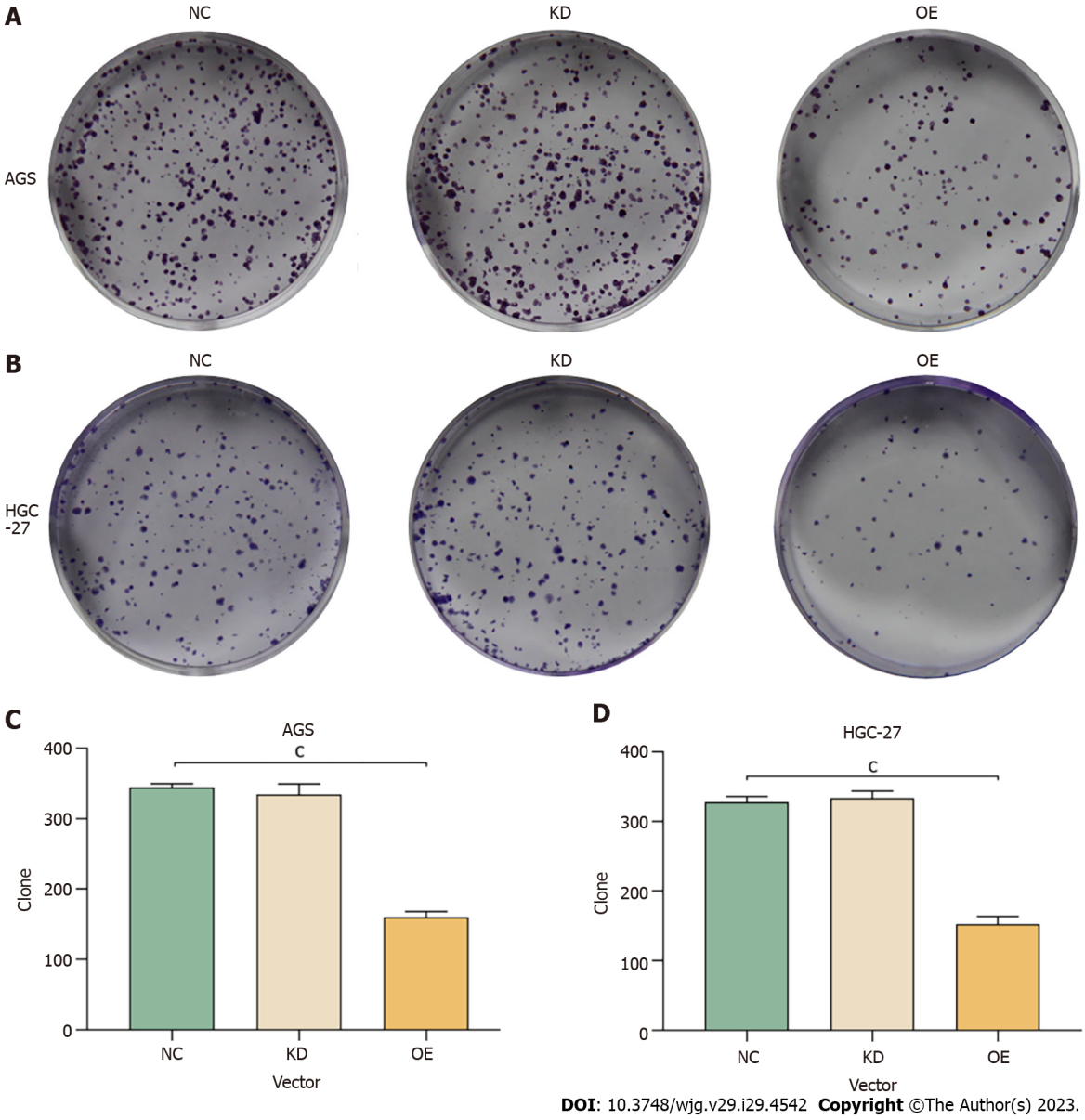

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Figure 3 Effect of miR-204-3p on colony forming ability in AGS and HGC-27 cells. A and B: Crystal violet staining diagram of AGS and HGC-27 cells; C and D: Statistical results of colony forming ability in AGS and HGC-27 cells. °P < 0.001. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

MiR-204-3p retarded GC cell cycle

We analyzed cell cycle distribution to investigate whether miR-204-3p inhibited GC cell proliferation by mediating the cell cycle (Figure 5). Among AGS cells, the G0/G1 phase percentages were 44.91 ± 1.15%, 45.36 ± 0.70% and 49.30 ± 0.41% in the NC, KD and OE groups, respectively (Figure 5A and C). Among HGC-27 cells, the G0/G1 phase percentages were 29.36 ± 0.29%, 29.57 ± 1.11% and 41.03 ± 0.47% in the NC, KD and OE groups, respectively (Figure 5B and D). These results revealed that AGS and HGC-27 cells with miR-204-3p overexpression had a notably higher number of cells in G0/G1 phase, which indicated that miR-204-3p overexpression blocked GC cell cycle in the G0/G1 phase.

MiR-204-3p inhibited the formation of subcutaneous tumor in BABL/c nude mice

A subcutaneous tumor formation experiment was conducted in BABL/c nude mice using lentivirus-transfected MKN-45 cells to observe the changes in tumor size and body weight. The results revealed that the OE group had smaller tumors compared to the NC group (Figure 6A and B). According to tumor growth data, it was found that back tumor growth was notably slower in the OE group compared to the NC group (Figure 6C). Additionally, the weight of BABL/c nude mice in the OE group increased significantly (Figure 6D), which indicated that miR-204-3p overexpression suppressed subcutaneous tumorigenesis in BABL/c nude mice.

Effect of miR-204-3p on the MAPK signaling pathway

We investigated the effect of miR-204-3p on the MAPK signaling pathway. Firstly, the impact of miR-204-3p on the

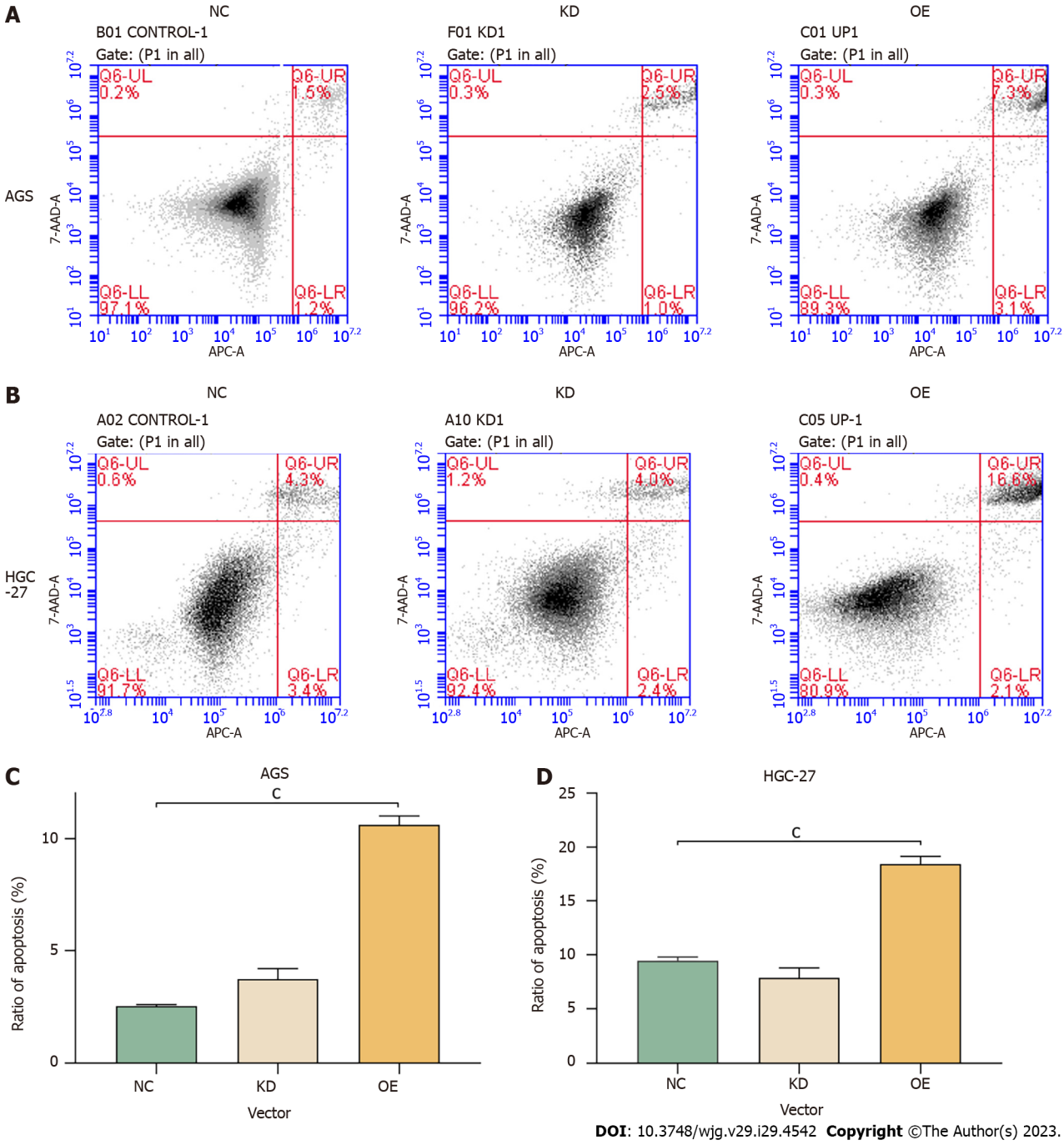

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Figure 4 Effects of miR-204-3p on apoptosis in AGS and HGC-27 cells. A and B: Flow cytometry was used to analyze apoptosis of AGS and HGC-27 cells; C and D: Statistical results of AGS and HGC-27 cell apoptosis. °$P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

mRNA levels of *KRAS*, *ERK1* and *ERK2* was detected using qRT-PCR. The results revealed that miR-204-3p overexpression resulted in a significant decrease in *KRAS*, *ERK1* and *ERK2* in AGS and HGC-27 cells (Figure 7A and B). We further investigated the impact of miR-204-3p on the MAPK signaling pathway-related proteins, including KRAS, ERK1/2, and p-ERK1/2. The results showed that in HGC-27 and AGS cells, miR-204-3p overexpression caused noteworthy lower levels of KRAS and p-ERK1/2, but no significant difference was observed in ERK1/2 (Figure 7C-F). These findings indicated that miR-204-3p overexpression effectively inhibited GC cell proliferation, and this effect was achieved through the inhibition of KRAS, which subsequently prevented the phosphorylation of downstream effector protein ERK1/2 in the MAPK signaling pathway.

Effects of miR-204-3p on apoptosis-related proteins

The effect of miR-204-3p on apoptosis-related proteins in GC cells was investigated. The results indicated a reduction in BCL-2 and the BCL-2/BAX ratio, as well as an increase in Caspase-3 in the OE group (Figure 8A-D), which confirmed that miR-204-3p overexpression stimulated GC cell apoptosis *via* the BCL-2/BAX signaling pathway.

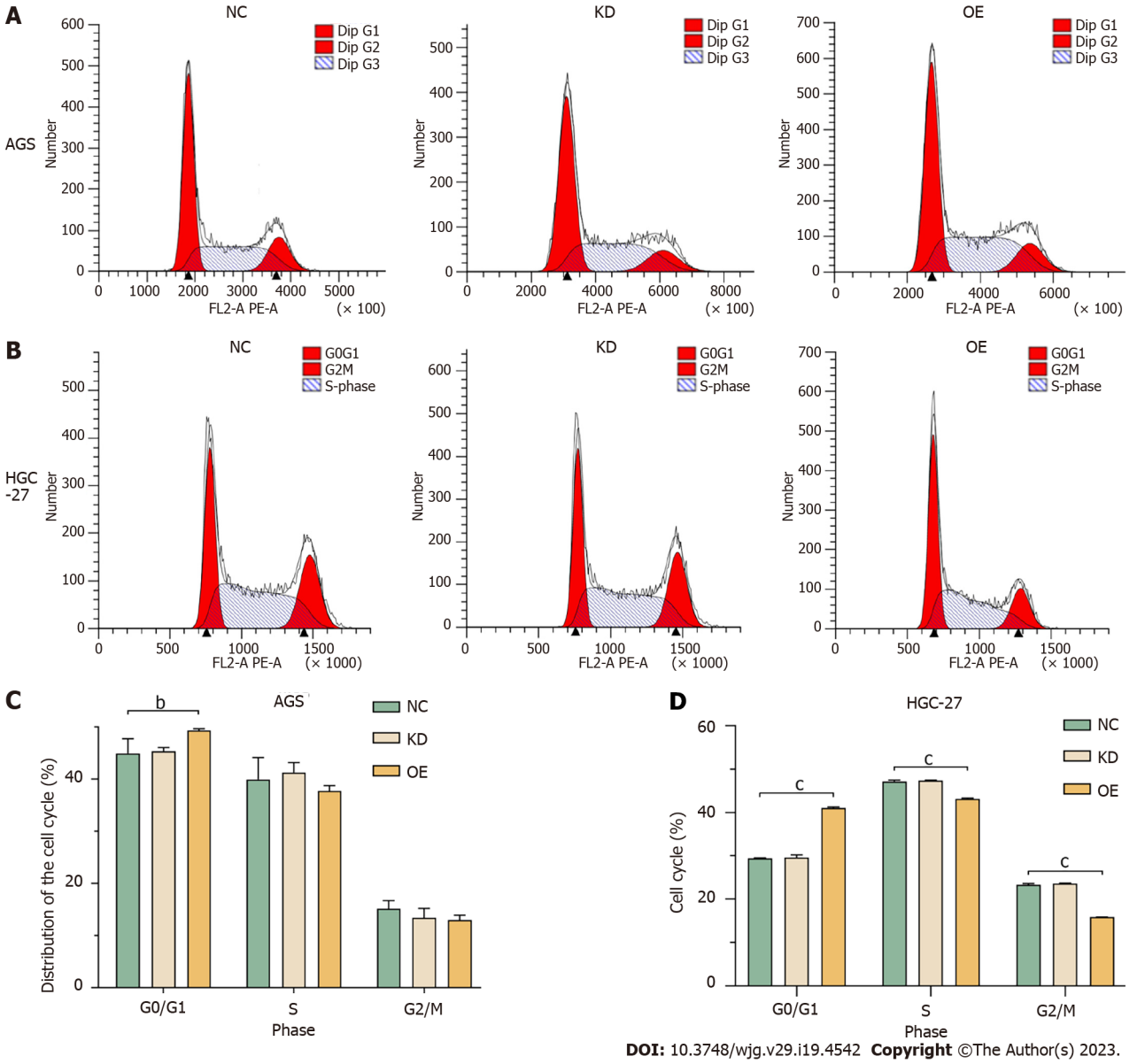

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Figure 5 Effects of miR-204-3p on the cell cycle in AGS and HGC-27 cells. A and B: Cell cycle analysis in AGS and HGC-27 cells by flow cytometry; C and D: Percentage of cell cycle distribution in AGS and HGC-27 cells. $^{b}P < 0.01$, $^{c}P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

Effect of miR-204-3p on necroptosis-related proteins

We examined the protein changes in RIP1, p-RIP1, MLK1 and p-MLK1 during necroptosis and investigated the impact of miR-204-3p on GC cells necroptosis. The results revealed that miR-204-3p overexpression significantly inhibited the protein expression levels of p-RIP1 and p-MLK1 (Figure 8E-H), which confirmed that miR-204-3p overexpression inhibited GC cells necroptosis *via* the RIP1/MLK1 signaling pathway.

DISCUSSION

MiR-204-3p plays a crucial role in various diseases. Some studies have confirmed that miR-204-3p upregulation can be targeted to inhibit Nox4 to reduce memory deficits[21]. Moreover, its overexpression inhibited high glucose induced lens epithelial cells migration and epithelial-mesenchymal transition (EMT), and inhibited high glucose induced podocytes apoptosis and dysfunction[13,15]. Notably, miR-204-3p upregulation inhibited malignant melanoma migration, invasion and EMT progression by targeting inhibition of PAX2[19], its upregulation also inhibited hepatocellular carcinoma cell proliferation by targeting inhibition of FN1[22]. MiR-204-3p was found to be underexpressed in bladder cancer tissues and was related to poor prognosis, it regulated bladder cancer cell proliferation by targeting LDHA mediated glycolysis [16]. Apoptosis of glioma cells is induced by miR-204-3p targeting IGFBP2[23]. Furthermore, LINC00963 was overexpressed in osteosarcoma tissues and was related to poor prognosis, miR-204-3p reversed LINC00963 in promoted

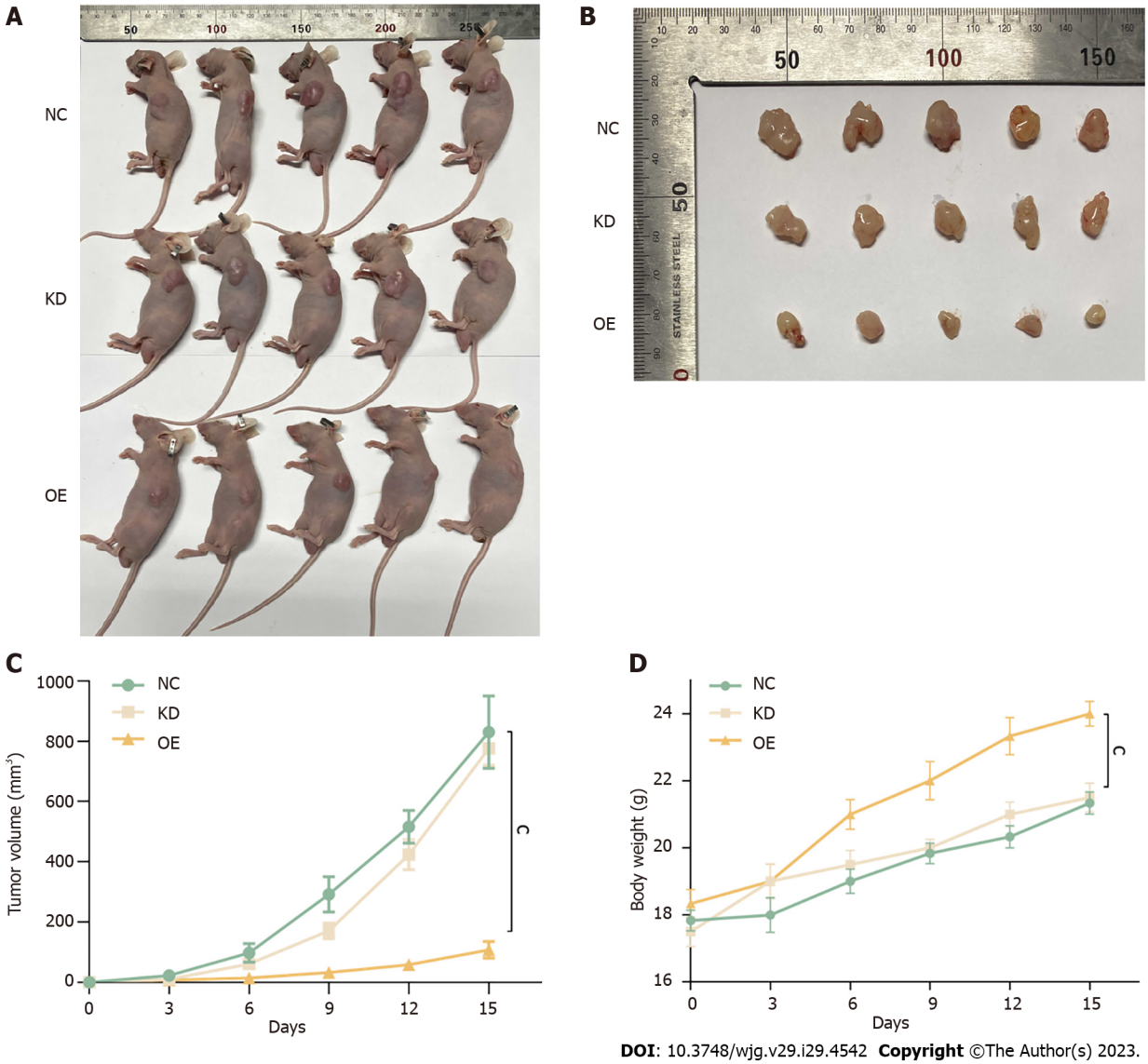

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Figure 6 Effect of miR-204-3p on tumor size in BABL/c nude mice. A: Comparison of gastric carcinoma tumor xenograft models in BABL/c nude mice; B: Comparison of subcutaneous tumors on the back in BABL/c nude mice; C: Statistical graph of tumor growth curve for gastric carcinoma tumor xenograft models in BABL/c nude mice; D: Statistical graph of body weight growth curve in BABL/c nude mice. $^{c}P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

osteosarcoma cell proliferation and inhibited migration and invasion[24]. LINC00514 was upregulated in GC tissues, its overexpression stimulated GC cell growth and inhibited EMT by sponging miR-204-3p/KRAS[20]. These results suggest that miR-204-3p may be a new target for cancer therapy.

Firstly, we found that the expression of miR-204-3p was low in GC cells, and its overexpression resulted in the inhibition of cell proliferation, colony formation ability, and the cell cycle, while promoting apoptosis. *In vivo* tumor formation experiments in 4-week-old BABL/c nude mice verified that miR-204-3p overexpression inhibited subcutaneous tumor growth. Thus, both *in vitro* and *in vivo* experiments demonstrated the inhibitory influence of miR-204-3p overexpression on GC cells.

Apoptosis, a programmed cell death, is a natural barrier against tumorigenesis. However, in cancer, abnormal expression of anti-apoptotic or pro-apoptotic proteins causes inhibition of apoptotic pathways[25]. For instance, BCL-2 functions as an anti-apoptotic protein, preventing cytochrome C liberation from mitochondria, thereby inhibiting apoptosis. On the other hand, BAX is a common pro-apoptotic protein that can form homodimers or bind to BCL-2 to form heterodimers that activate Caspase-3 to promote apoptosis[26]. The BCL-2/BAX ratio is closely related to tumor progression, and a lower ratio indicates a stronger apoptosis effect. Therefore, most anti-cancer drugs rely on the BCL-2/BAX mechanism to kill cancer cells[27]. This study verified that miR-204-3p overexpression led to the upregulation of Caspase-3 in GC cells, while downregulating BCL-2 and the BCL-2/BAX ratio. These findings provide evidence that miR-204-3p overexpression can stimulate apoptosis and inhibit GC cell proliferation through the BCL-2/BAX/Caspase-3 apoptosis pathway.

In tumors, the MAPK pathway is frequently activated to control apoptosis, cell growth, and cell division[28]. Signal transmission of the MAPK signaling pathway follows a three-step enzyme-linked reaction. KRAS, as an upstream

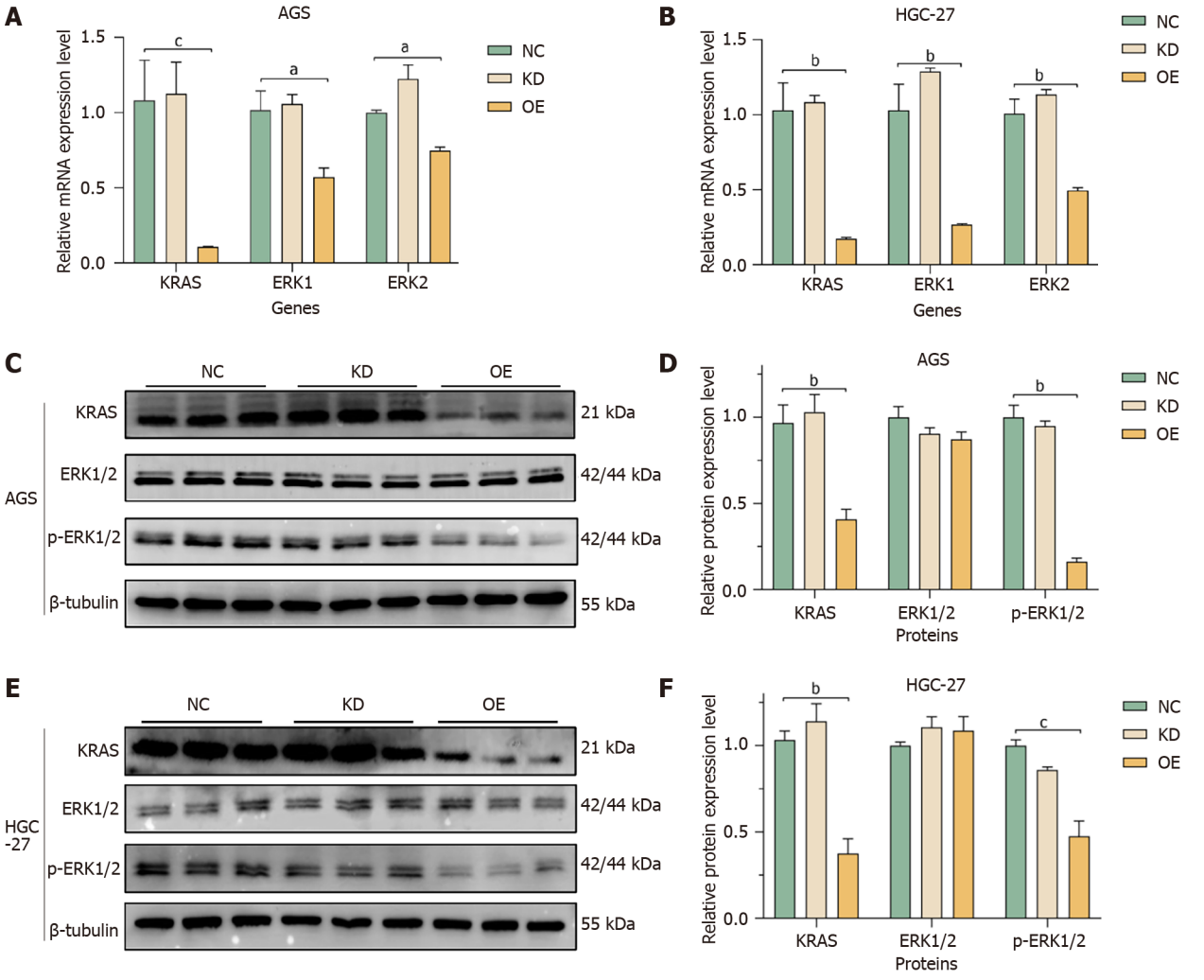

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Figure 7 Effect of miR-204-3p on the MAPK signaling pathway in AGS and HGC-27 cells. A and B: Effects of miR-204-3p on mRNA levels of *KRAS*, *ERK1* and *ERK2* in AGS and HGC-27 cells; C and D: Effects of miR-204-3p on protein levels of *KRAS*, *ERK1/2* and *p-ERK1/2* in AGS cells; E and F: Effects of miR-204-3p on protein levels of *KRAS*, *ERK1/2* and *p-ERK1/2* in HGC-27 cells. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

activation protein, is activated when bound to GTP. This change causes the recruitment of KRAS to RAF on the cell membrane and promotes RAF activation. Activated RAF phosphorylates and activates MEK, while MEK phosphorylates and further activates ERK, which is located at the end of the signaling pathway and can transfer into the nucleus and bind to transcription factors, thereby regulating transcription programs and mediating cell growth, migration and differentiation[29-31]. Previously, we established that miR-204-3p targeted KRAS[20]. The current study confirmed that miR-204-3p upregulation can inhibit KRAS and p-ERK1/2, which suggested that miR-204-3p overexpression could inhibit the MAPK signaling pathway.

Necroptosis is a newly discovered mechanism of programmed cell death that has the potential to regulate tumorigenesis[32]. This process is primarily regulated by three proteins: RIP1, RIP3 and MLK1. Specifically, RIP1 is activated through phosphorylation, which then recruits RIP3[33]. Once activated, phosphorylated RIP3 can oligomerize MLK1 and transfer it to the plasma membrane, ultimately resulting in necroptosis characterized by cell swelling and organelle damage[34,35]. Interestingly, necroptosis has been found to both promote and inhibit cancer growth. As a form of cell death, necroptosis inhibits the development of tumors, yet it may also incite an inflammatory reaction that encourages cancer metastasis and immunosuppression. Research has revealed that glioblastoma, pancreatic cancer, and lung cancer can be impacted by the upregulation of RIP1, RIP3, and MLK1[36-38]. It was found that downregulation of MLK1 inhibited tumor cell growth and increased sensitivity to radiotherapy in both GC and ovarian cancer[39,40]. We detected necroptosis-related proteins and discovered that miR-204-3p overexpression decreased p-RIP1 and p-MLK1. These findings suggest that miR-204-3p overexpression can inhibit necroptosis through the RIP1/MLK1 pathway, ultimately inhibiting GC cell proliferation.

To sum up, our study verified that miR-204-3p is underexpressed in GC, and that its overexpression inhibits GC cell proliferation, promotes apoptosis, arrests the cell cycle in the G0/G1 phase, inhibits cell colony formation and the formation of subcutaneous tumors. Necroptosis is typically initiated by tumor necrosis factor (TNF) stimulation[41]. RIP1 binds to FADD, which then recruits caspase-8. The activation of caspase-8 promotes the process of RIP1-dependent

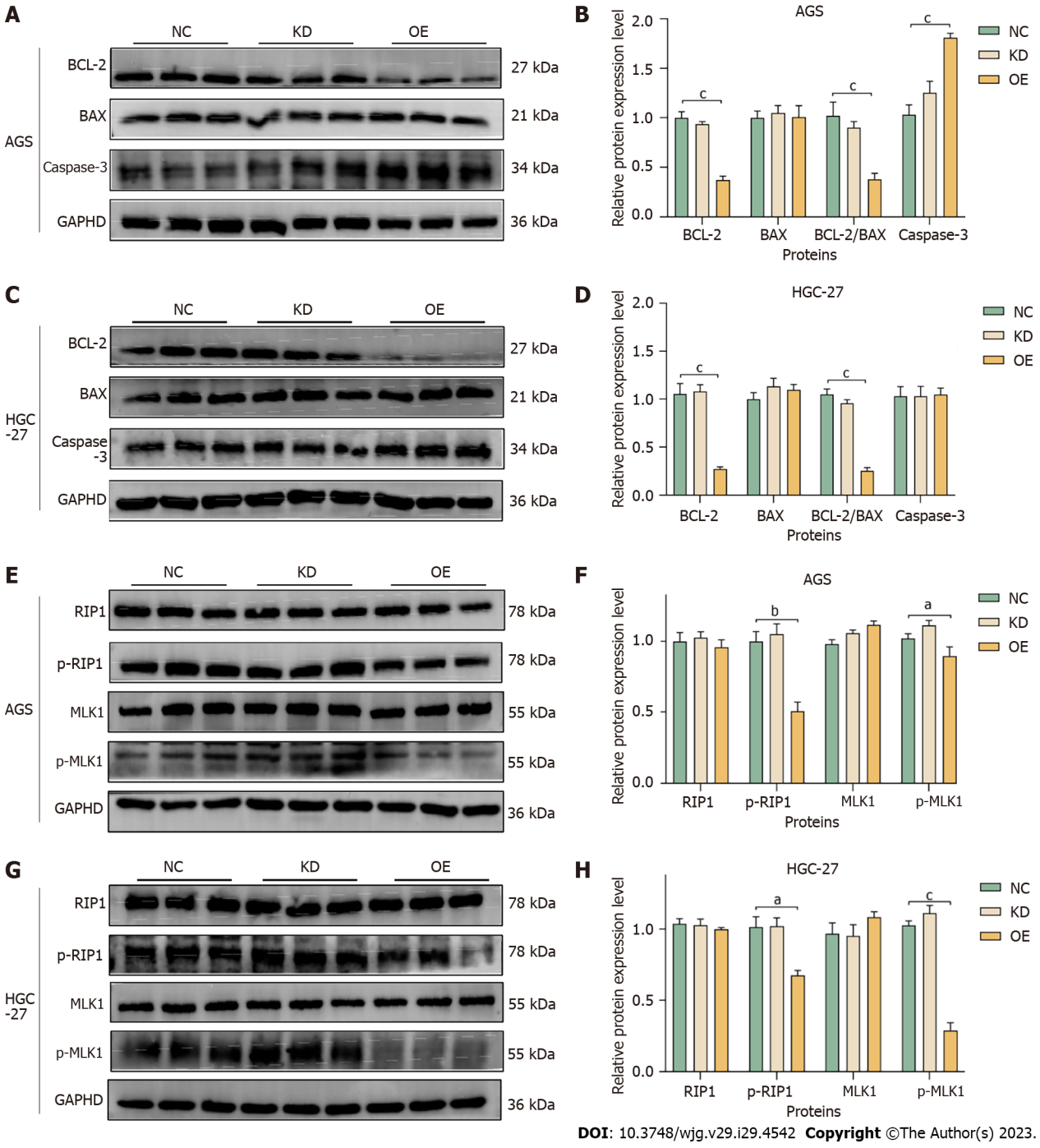

Figure 8 Effects of miR-204-3p on apoptosis-related proteins and necroptosis-related proteins in AGS and HGC-27 cells. A and B: Effect of miR-204-3p on apoptosis-related proteins BCL-2, BAX and Caspase-3 in AGS cells; C and D: Effect of miR-204-3p on apoptosis-related proteins BCL-2, BAX and Caspase-3 in HGC-27 cells; E and F: Effects of miR-204-3p on necroptosis-related proteins RIP1, p-RIP1, MLK1, and p-MLK1 in AGS cells; G and H: Effects of miR-204-3p on necroptosis-related proteins RIP1, p-RIP1, MLK1, and p-MLK1 in HGC-27 cells. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

apoptosis[32]. Additionally, RIP1 activates ERK to regulate the MAPK signaling pathway[42-44]. In the MAPK signaling pathway, phosphorylation of ERK can activate BCL-2, which in turn stimulates the apoptosis pathway and accelerates the process of apoptosis[45,46]. We verified that miR-204-3p overexpression can inhibit GC cell proliferation by inhibiting the MAPK signaling pathway and inhibiting the RIP1/MLK1 necroptosis pathway to promote the BCL-2/BAX/Caspase-3 apoptosis pathway (Figure 9).

CONCLUSION

MiR-204-3p overexpression inhibited GC cell proliferation by inhibiting the MAPK pathway and necroptosis pathway to

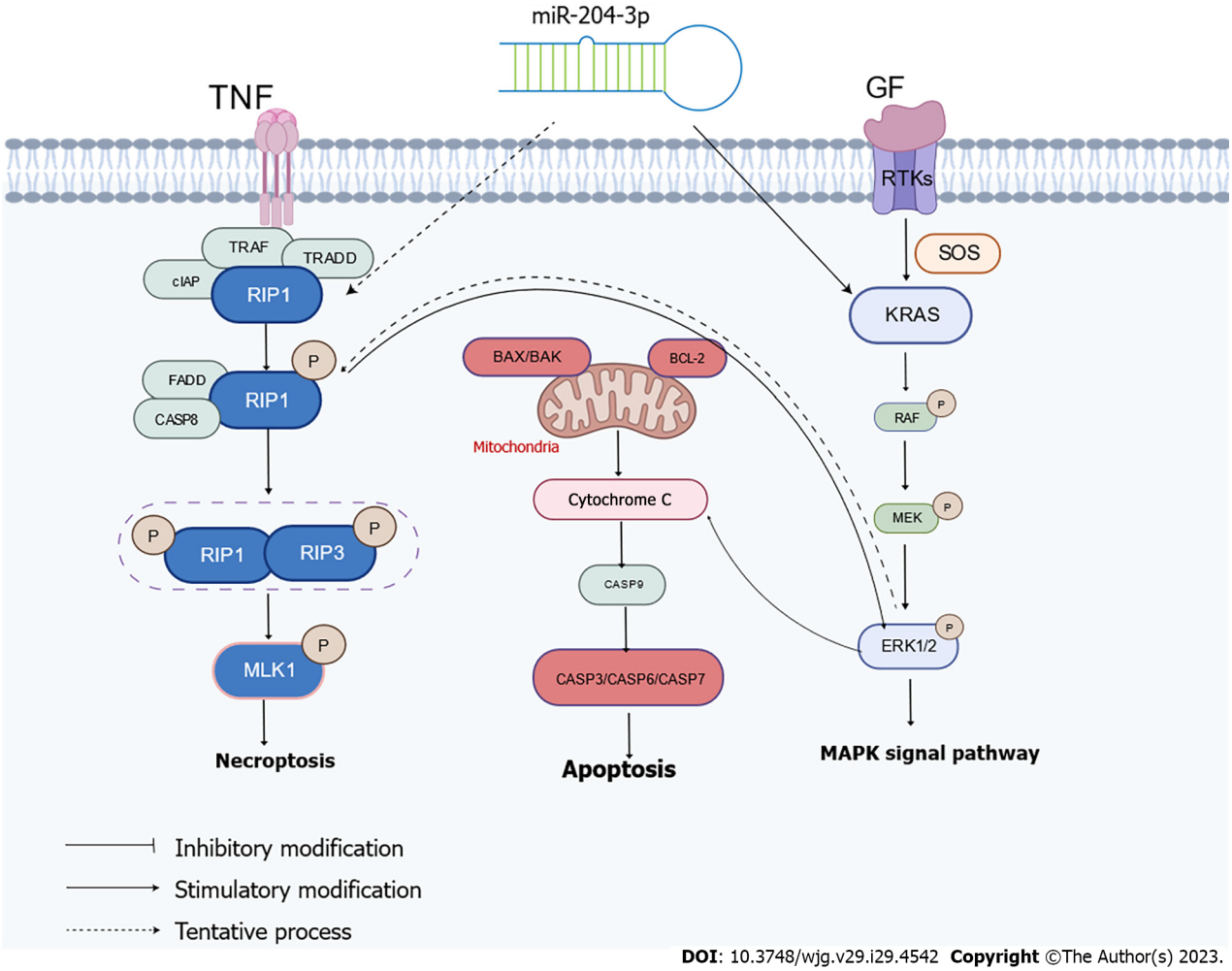

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Figure 9 Mechanism of the effect of miR-204-3p on the MAPK signaling pathway, apoptosis and necroptosis. TNF: Tumor necrosis factor; GF: Germ-free.

promote GC cell apoptosis. Thus, miR-204-3p may represent a new potential therapeutic target for GC.

ARTICLE HIGHLIGHTS

Research background

Gastric carcinoma (GC) is a common gastrointestinal malignancy worldwide. Based on the cancer-related mortality, the current prevention and treatment strategies for GC still show poor clinical results. Therefore, it is important to find effective treatment targets.

Research motivation

At present, the main treatment for GC is surgery, chemotherapy and radiotherapy, but the therapeutic effect is not ideal.

Research objectives

To explore the effect of miR-204-3p on GC cells.

Research methods

We determined the expression level of miR-204-3p in GC, and then used an miR-204-3p overexpression vector and an miR-204-3p knockdown vector in GC cells. The influence of miR-204-3p on the changes in cell phenotype and tumorigenicity *in vivo* was assessed. Furthermore, the effects of miR-204-3p on various proteins related to the MAPK signaling pathway, necroptosis signaling pathway and apoptosis signaling pathway in GC cells were investigated.

Research results

It was found that miR-204-3p was underexpressed in GC, and miR-204-3p overexpression inhibited GC cell viability, promoted cell apoptosis, blocked the cell cycle, inhibited colony formation ability and inhibited tumorigenicity *in vivo*. It

was also verified that miR-204-3p overexpression can promote apoptosis by inhibiting the MAPK pathway and the necroptosis pathway, thus inhibiting GC cell proliferation.

Research conclusions

MiR-204-3p overexpression inhibited GC cell proliferation by inhibiting the MAPK pathway and the necroptosis pathway to promote GC cell apoptosis.

Research perspectives

MiR-204-3p can be used for targeted therapy of GC, and can also be used as a new biomarker for GC.

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FOOTNOTES

Author contributions: Li X carried out most of the studies, analyzed the data, and wrote the manuscript; Nan Y designed the study and revised the manuscript; Tibenda JJ, Du YH and Huang SC wrote the manuscript, and conducted the chart-making work; Ning N and Chen GQ were responsible for the total transcriptomic; Yang YT and Meng FD performed parts of the *in vivo* and *in vitro* experiments, and conducted statistical analysis of the data; Yuan L and Nan Y provided the conceptual and technical guidance and revised the manuscript critically for important intellectual content; all authors have read and approved the manuscript.

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