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MicroRNA-668-3p Mediates Macrophage M2 Polarization by Targeting NFKBIA to Affect Gastric Cancer Cell Proliferation and Migration

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Macrophage polarization is implicated in the pathological mechanism of gastric cancer (GC). This study investigated how the miR-668-3p/ Nuclear factor kappa B inhibitor alpha (NFKBIA) axis drives macrophage polarization to contribute to GC progression. Inhibitors or shRNA were used to interfere with the expression of miR-668-3p or NFKBIA in the GC cell line. Subsequently, CCK-8, EdU, wound healing, and transwell assays were used to assess the biological behavior of the GC cells. Bioinformatics analysis predicted the target connection between miR-668-3p and NFKBIA, and a dual luciferase reporter gene experiment confirmed this relationship. After THP-1 macrophages were co-cultured with the supernatant of transfected GC cells, the M1 and M2 macrophage phenotypes were determined. Subsequently, these THP-1 macrophages were co-cultured with GC cells using the Transwell, and the biological behaviors of the GC cells were determiend. miR-668-3p inhibitor suppressed proliferation, invasion and migration of GC cells. The phenotype of M1 macrophage (IL-1β, TNF-α and IL-6) was boosted yet the phenotype of M2 macrophage (CD206, Fizz1 and IL-10) was declined by miR-668-3p inhibitor. NFKBIA was the target gene of miR-668-3p and it reversed the effects of miR-668-3p inhibitor on macrophage polarization and biological behaviors of the GC cells. miR-668-3p suppressed NFKBIA in GC cells to mediate M2 polarization of macrophages, thereby facilitating the tumorigenesis of GC.

Keywords: gastric cancer; macrophage polarization; metastasis; miR-668-3p; nuclear factor kappa B inhibitor alpha
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Introduction

Gastric cancer (GC) is one of the most prevalent malignant tumors in the world, with an annual incidence of 990,000 people worldwide, of which about 738,000 people die (Machlowska et al. 2020). Geographical variations exist in the occurrence of GC, in East Asia, the incidence is higher than in North America and Northern Europe (Guan et al. 2023). It is worth noting that although the incidence of GC increases with age, it tends to be younger due to changes in diet structure, increased work pressure and Helicobacter pylori infection, and 10% of GC is still found under 45 years old (Joshi and Badgwell 2021). Although the incidence of GC has decreased in most parts of the world in the past few decades, and the 5-year survival rate of early GC exceeds 95% (Song et al. 2017), most GC patients have metastasized at the time of diagnosis because of the lack of effective early diagnosis technology, and the median survival rate is less than one year (Patel and Cecchini 2020). To enhance the treatment status of GC patients, it is therefore imperative to gain additional insight into the pathophysiology of GC and identify pertinent targets for diagnosis and treatment.

People are becoming more and more fascinated with

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the involvement of macrophages in GC since they are one of the immune cells in the tumor microenvironment and have dual effects of promoting and inhibiting tumor growth (Gambardella et al. 2020). Because of their versatility, macrophages can exhibit both pro- and anti-inflammatory (M1 and M2) phenotypes (Boutilier and Elsawa 2021). It has been suggested that GC formation may be aided by macrophage M2 polarization (Li et al. 2019). It is important to remember that macrophages can shift their phenotype from M1 to M2 in response to external stimuli or changes in their surroundings. For instance, hypoxia and lactic acid can drive M2 polarization of macrophages (Murray 2017). Nuclear factor kappa B inhibitor alpha (NFKBIA), the coding gene of IκB, the key node of NF-κB pathway, has been proved to regulate macrophage activation and thus inhibit the progression of inflammatory reaction (Zhang et al. 2023). Furthermore, NFKBIA functions as a tumor suppressor in a variety of malignancies, including gliomas, lung cancer, and cervical cancer (Miyar et al. 2016; Chen et al. 2021; Yao et al. 2023). Notably, Li et al. (2017) reported that NFKBIA single nucleotide polymorphism is related to GC susceptibility. Therefore, we speculate that NFKBIA may regulate the polarization of macrophages to affect the progression of GC.

MicroRNA (MiRNA) is involved in numerous biological processes and has the ability to influence gene expression through interactions with downstream target genes (Hill and Tran 2021). Research has indicated that miRNA exhibits remarkable stability in tissue and circulation expression, making it a potential biomarker for GC diagnosis and prognosis (Jelski and Mroczko 2022). MiR-668-3p has been reported to be overexpressed in GC and is related to the carcinogenesis of GC (Li et al. 2023). Moreover, we found that miR-668-3p has a targeted binding relationship with NFKBIA through starBasewebsite (https://rnasysu.com/encori/) prediction. Consequently, we hypothesize that miR-668-3p may inhibit the expression of NFKBIA and mediate M2 polarization of macrophages in tumor microenvironment to promote the proliferation and migration of GC, thus affecting the GC progression.

Materials and Methods

Cell culture

GC cell lines MKN45 and AGS (SUNNCELL, Wuhan, China) were cultured in Dulbecco's modified eagle medium (DMEM) complete medium (SUNNCELL). THP-1 cells (Pricella, Wuhan, China) were cultured in RPMI-1640 complete medium supplemented with 0.05 mM β-mercaptoethanol (Pricella). Cells were placed in an incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO_2. In order to obtain THP-1 macrophages, THP-1 cells were incubated with 100 ng/mL Phorbol 12-myristate 13-acetate (PMA, MedChemExpress, Shanghai, China) for 72 h, as described above (Dabla et al. 2022).

Cell transfection

miR-668-3p mimic, mimic-negative control (NC), inhibitor and inhibitor-negative control (NC) were ordered from Ribobio (Guangzhou, China). NFKBIA specific short hairpin RNA (shNFKBIA, target sequence: 5′-CCGAGACTTTCGAGGAAATAC-3′) was ordered from Santa cruz (Dallas, Texas, USA). Before transfection, the cells were digested by trypsin, inoculated and cultured for 24 h until the confluence degree of 60-70% was reached. Following their dilution in opti-MEM (Thermo Fisher Scientific), the transfection reagent (Thermo Fisher Scientific) and the plasmid/oligonucleotide fragment were mixed. The incubated mixture was delivered to cells, and then transfection efficiency was evaluated after 24 h.

Cell treatment

There were three parts in the cell experiment. In the first part, GC cells were transfected with miR-668-3p inhibitor or inhibitor-NC. In the second part, GC cells transfected inhibitor-NC, miR-668-3p inhibitor or co-transfected miR-668-3p inhibitor and shNFKBIA, and then the supernatants of transfected GC cells were co-cultured with THP-1 macrophages as described above (Ma et al. 2024). In the third part, transfected GC cells (5 × 105 cells/well) were seeded in the lower chamber of Transwell, while the second part of THP-1 macrophages were seeded in the upper chamber of Transwell as described above (Yuan et al. 2020).

Quantitative real-time PCR (qPCR) assay

GC cells or THP-1 macrophages were treated with an RNA extraction kit (Agbio, Changsha, China) to extract total RNA, which was then measured using ultraviolet spectrometry at 260 nm. The complementary DNA (cDNA) was created by reverse-transcription of the RNA using the cDNA Synthesis Master Mix (Agbio). With the use of Probe qPCR Mix (Agbio) and a Real-Time PCR instrument (Thermo Fisher Scientific), the quantification of the target genes was examined by qPCR. The internal reference was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6. The $2^{-\Delta\Delta Ct}$ technique was used to calculate the results. The primer sequences were listed in Table 1.

Cell counting kit-8 (CCK-8)

We adopted the CCK-8 kit (PHYGENE, fuzhou, China) to assess the viability of the cell. Cells were inoculated into 96-well plates (5×10^3 per well). After the cells were given the proper care according to the aforementioned groupings, they were permitted 48 h to grow. Subsequently, 10 μL of CCK-8 solution was added to the cells, and they were incubated for 4 h under light protection. Next, using a BioTek microplate reader (Winooski, VT, USA), the absorbance at a wavelength of 450 nm was calculated.

5-ethynyl-2′–deoxyuridine (EdU) assay

After the cells were cultivated in 6-well plates, the

Table 1. Primers used in this study.

Genes	5'--> 3'
miR-668-3p Forward	TGTCACTCGGCTCGGCC
miR-668-3p Reverse	GTGCAGGGTCCGAGGT
NFKBIA Forward	TGTGCTTCGAGTGACTGACC
NFKBIA Reverse	TCACCCCACATCACTGAACG
IL-1β Forward	AACCTCTTCGAGGCACAAGG
IL-1β Reverse	AGATTCGTAGCTGGATGCCG
TNF-α Forward	GCCAGCCTTCATCCACTCTC
TNF-α Reverse	GGGAACTGTTGGGGAGAAGG
IL-6 Forward	GTCCAGTTGCCTTCTCCCTGG
IL-6 Reverse	CCCATGCTACATTTGCCGAAG
CD206 Forward	GCCTCGTTGTTTTGCGTCTT
CD206 Reverse	GAGAACAGCACCCGGAATGA
Fizz1 Forward	CCTCTTGCCTCCTTCTCATCC
Fizz1 Reverse	CTTGGTTGGGACCCTGGTTT
IL-10 Forward	ACACATCAGGGGCTTGCTC
IL-10 Reverse	GTGGTCAGGCTTGGAATGGA
GAPDH Forward	AGGAAATGATGACCTCCTGAACT
GAPDH Reverse	GAAGATGCGGTCACCTCACA

aforementioned grouping procedure was applied. After preparing the working solution in accordance with the EdU kit (Beyotime, Shanghai, China) instructions, it was added to the 6-well plate. Following a two-hour incubation period, the cells underwent sequential permeabilization and fixation. Following the addition of the Click solution, the cells were permitted to rest in the dark for 30 min. DAPI was used to stain the nucleus. Finally, a fluorescent microscope (Nikon, Tokyo, Japan) was used to examine the cells.

Wound healing assay

A pipette was used to make scratches on the GC cell monolayer. The PBS was used to eliminate the floating cells. After the scratch, the cell migration area was measured both immediately (A area) and 24 h later (B area). The migration rate was calculated as (A-B)/A*100%.

Transwell assay

To investigate the GC cells' capacity to invade, Transwell inserts were pre-coated with Matrigel (Corning, NewYork, USA). The serum-depleted GC cells were inserted into the inserts. To foster cell invasion, a medium containing 20% FBS was injected to the basolateral chamber. After 24 h, the invaded cells were stained with 0.1% crystal violet (Weikeqi-biotech, Chengdu, China), and the non-invaded cells were removed with a cotton swab. The invaded cells were then kept in 4% paraformaldehyde. Results were viewed using an inverted microscope (Yongxin, Ningbo, China).

Western blot

After the protein was extracted using RIPA buffer (Weikeqi-biotech), the total protein was measured using the Protein Quantitative Kit (Life-iLab, Shanghai, China). After loading the proteins onto SDS-PAGE gels (Life-iLab), the proteins were transferred to PVDF membranes (Weikeqi-biotech). Using BSA (Life-iLab), the membranes were blocked. The membranes were treated with the secondary antibody (Table 2) for two hours at room temperature after being incubated with the primary antibody (Table 2) for the entire night at 4°C. Employing an iBright FL1500 Imaging System (Thermo Fisher Scientific), the membranes were ultimately identified after being exposed to the ECL Western Blotting Substrate (Life-iLab). β-actin was the internal control used in this experiment.

Dual luciferase reporter gene (DLRG) assay

miRDB (https://mirdb.org/) was used to predict the potential binding site between NFKBIA and miR-668-3p. The GC cells were obtained when 70% confluence had been reached after a 24-h culture in 24-well plates. Next, we constructed reporter plasmids for NFKBIA-wild type (NFKBIA-WT) and NFKBIA mutant (NFKBIA-MUT) using the pGL3 Luciferase Reporter Vector (Promega, WI, USA). Then, employing Lipofectamine 3000 transfection reagent, the cells were co-transfected with miR-668-3p inhibitor or inhibitor-NC and NFKBIA-WT or NFKBIA-MUT reporter plasmids, as well as pRL Renilla Luciferase Control Reporter Vectors (Promega). After 48 h, the Dual-luciferase Reporter Assay System (Promega) was used to assess the activity of firefly and renilla luciferase using a GloMax 96 Microplate Luminometer.

Statistical analysis

Statistical analysis was undertaken using GraphPad Prism software (Version 8.0, USA). The measurement data

Table 2. Antibodies used in this study.

Name	Catalog	Dilution	Manufacturer
NFKBIA	ab32518	1/1000	
IL-1β	ab315084	1/1000	
TNF-α	ab183218	1/1000	
IL-6	ab233706	1/1000	
CD206	ab125028	1/5000	Abcam, Cambrige, MA, USA
Fizz1	ab39626	1/1000	
IL-10	ab133575	1/1000	
β-actin	ab8227	1/2000	
goat anti rabbit	ab205718	1/10000	

were shown as mean ± standard deviation (SD). Multiple groups were done through a one-way analysis of variance followed by Tukey's *post hoc* test. In all statistical analysis, a level of significance of $p < 0.05$ was assumed.

Results

miR-668-3p facilitates proliferation, invasion and migration of GC cells

GC cells were successfully transfected with miR-668-3p inhibitor or inhibitor-NC, and it showed that miR-668-3p inhibitor could substantially suppress the levels of miR-668-3p in GC cells (Fig. 1A, $p < 0.05$). Next, miR-668-3p inhibitor had an impact on GC cells' biological behavior. It's interesting to note that miR-668-3p inhibitor prevented GC cells from proliferating, migrating, invading, and remaining viable (Fig. 1B-E, $p < 0.01$). The aforementioned findings indicate that miR-668-3p exerted the promoting effects on proliferation, invasion and migration of gastric cancer cells.

miR-668-3p targets NFKBIA to regulate NFKBIA expression

When miR-668-3p inhibitor was added, the expression level of NFKBIA in GC cells decreased significantly (Fig. 2A, $p < 0.05$). Compared with the inhibitor NC group, the relative luciferase activity of NFKBIA-Wt was significantly higher in the miR-668-3p inhibitor group, and compared with the mimic NC group, the relative luciferase activity of NFKBIA-Wt was significantly lower in the miR-668-3p mimic group, whereas the relative luciferase activity of NFKBIA-Mut did not have significant change in the relative luciferase activity of NFKBIA-Wt in the mimic group (Fig. 2B, $p < 0.05$). The results presented above indicate that MiR-668-3p has the ability to target and suppress the expression of NFKBIA.

miR-668-3p regulates M2 macrophage polarization by inhibiting NFKBIA

To ascertain the impact of the miR-668-3p/NFKBIA axis on macrophage polarization, we co-cultured THP-1 macrophages and GC cells co-transfected with miR-668-3p inhibitor/ inhibitor-NC and shNFKBIA. GC cells transfected with miR-668-3p inhibitor rather than shNFKBIA lowered the expression of miR-668-3p in macrophages (Fig. 3A, $p < 0.05$). Similarly, miR-668-3p inhibitor transfection of GC cells facilitated the expression of NFKBIA in THP-1 macrophages; co-transfection of miR-668-3p inhibitor and shNFKBIA reversed this promotion in NFKBIA expression in THP-1 macrophages (Fig. 3B, $p < 0.05$). Additionally, the addition of miR-668-3p inhibitor boosted the expression of M1-related factors (IL-1β, TNF-α and IL-6) while diminishing the expression of M2-related factors (CD206, Fizz1 and IL-10) (Fig. 3C-D, $p < 0.05$). Nevertheless, the influence of M2-related and MI-related variables by miR-668-3p inhibitor was reversed by shNFKBIA. The aforementioned findings illustrate that miR-668-3p suppresses NFKBIA expression to regulate M2 macrophage polarization.

miR-668-3p/NFKBIA axis regulates M2 macrophage polarization to facilitate proliferation, invasion and migration of GC cells

The biological functions of GC cells co-cultured with THP-1 macrophages were investigated. The vitality, proliferation, migration, and invasion of GC cells diminished with the addition of miR-668-3p inhibitor; this effect was somewhat counteracted by shNFKBIA (Fig. 4A-D, $p < 0.05$). These results imply that via blocking NFKBIA, miR-668-3p boosts the growth, invasion, and migration of GC cells.

Discussion

An increasing number of studies demonstrate that immune cell infiltration is essential for the generation and progression of GC; in particular, tumor-associated macrophages stimulate tumor activity by suppressing the immune system and promoting angiogenesis (Chen et al. 2023). This study investigated how the miR-668-3p/NFKBIA axis drives macrophage polarization to contribute to GC progression.

First, we investigated at how miR-668-3p affected the biological behavior of GC cells. MiR-668-3p has been shown to have pro-cancer impacts in the past. For example, it has been shown to target BTG2 in order to foster the proliferation of hepatocellular carcinoma and to target ID4 to

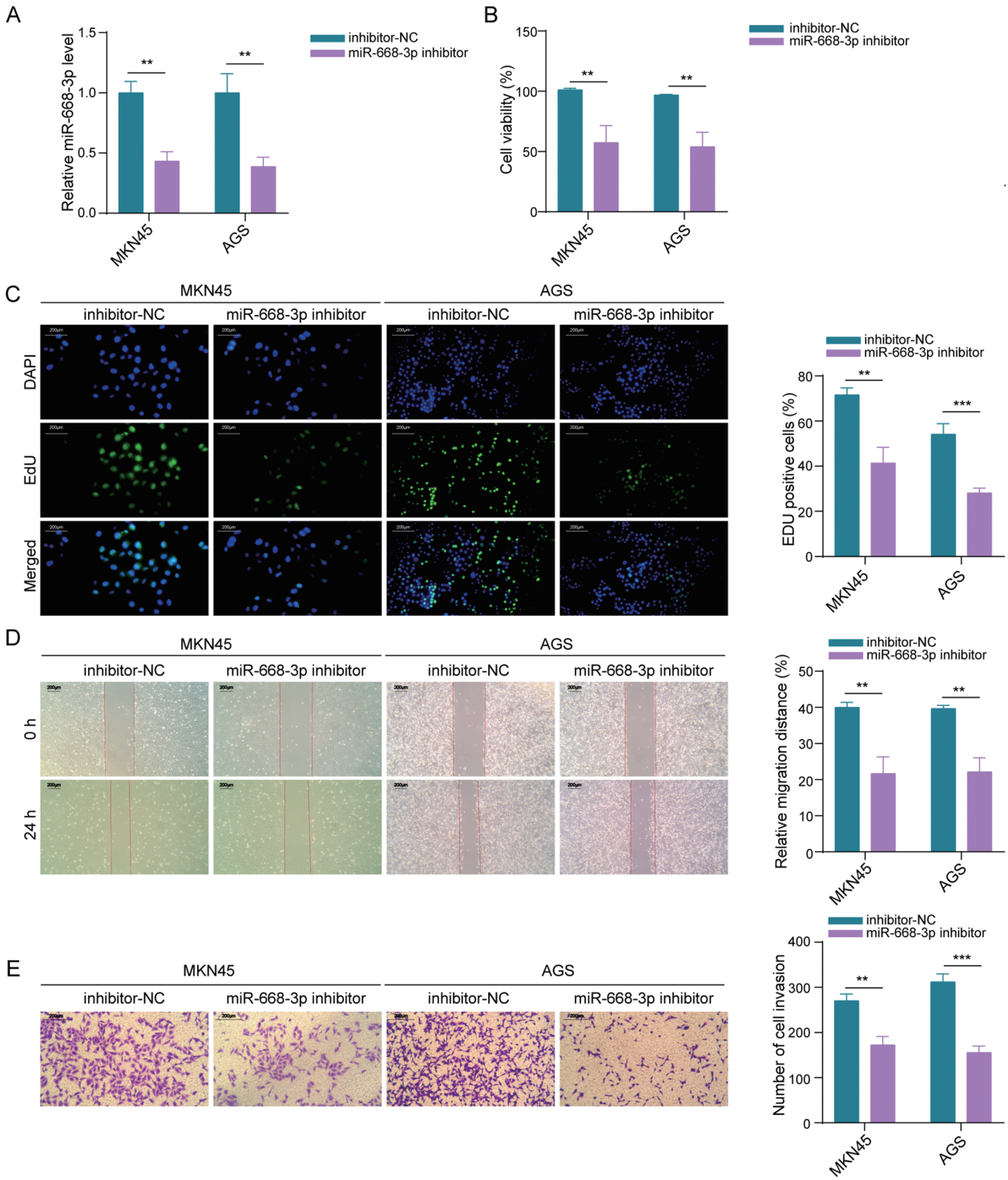

Fig. 1. miR-668-3p facilitates proliferation, invasion and migration of gastric cancer cells.
(A) The expressions of miR-668-3p in gastric cancer cells was determined by qPCR. (B) The viability of gastric cancer cells was determined by cell counting kit-8 (CCK-8). (C) The proliferation of gastric cancer cells was determined by EdU staining. (D) The migration of gastric cancer cells was determined by wound healing assay. (E) The invasion of gastric cancer cells was determined by transwell assay. Data were displayed as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

accelerate the growth and metastasis of colorectal cancer (Ma et al. 2020; Feng et al. 2022). Notably, via targeting SOCS3, miR-668-3p boosted GC formation both in vivo and in vitro (Li et al. 2023). Additionally, miR-668-3p inhibitor can inhibit the proliferation, invasion and migration of GC cells by inhibiting M2 polarization of THP-1

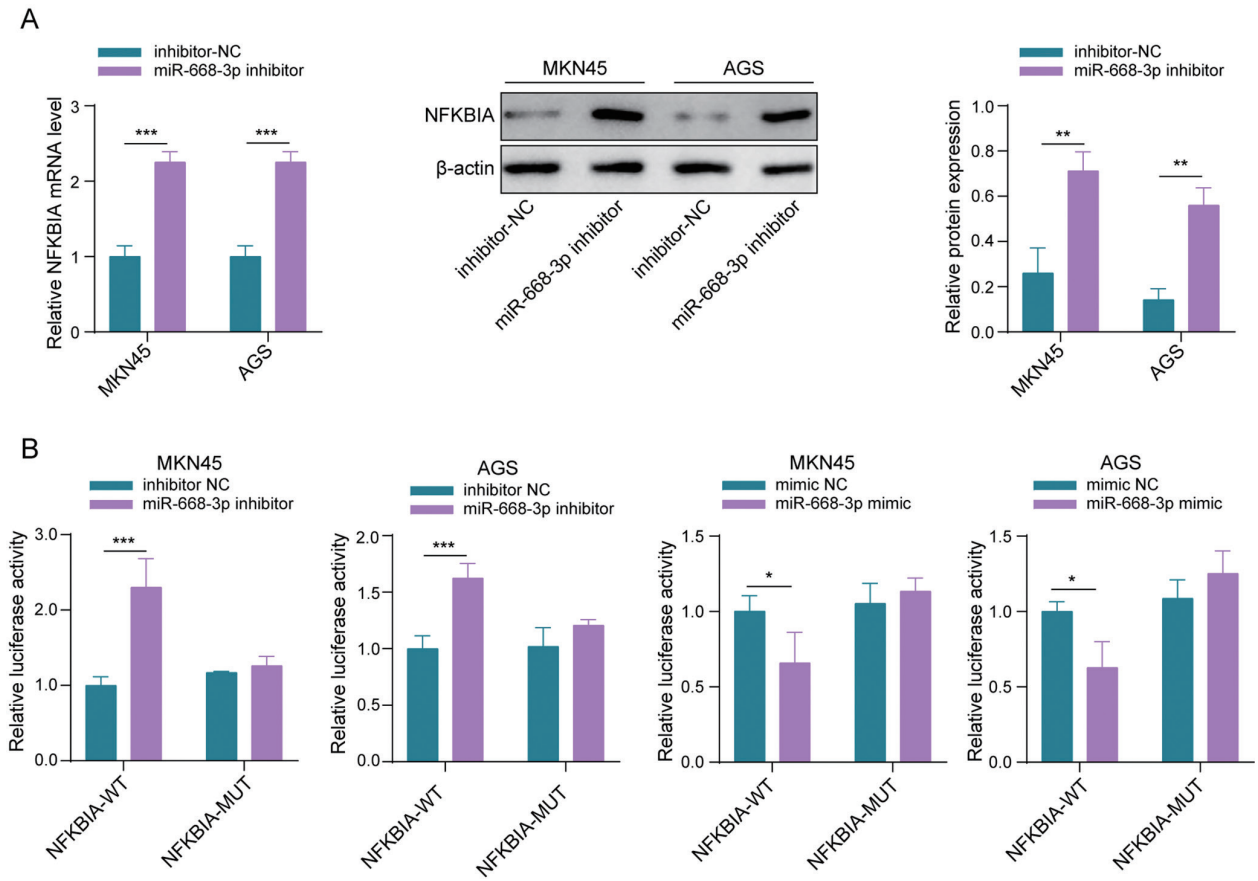

Fig. 2. miR-668-3p targets NFKBIA to regulate NFKBIA expression.
(A) The mRNA and protein levels of NFKBIA in gastric cancer cells was determined by qPCR and western blot. (B) The relationship between miR-668-3p and NFKBIA was determined by dual luciferase reporter gene assay. Data were displayed as mean ± SD. *p < 0.05, ***p < 0.001.

macrophages, which is in line with Li's research findings. Nonetheless, miR-668-3p has the ability to target NFKBIA, which is a novel finding to date.

It has been documented how NFKBIA functions in GC. According to Lu et al. (2020) NFKBIA up-regulation can prevent GC cell proliferation and xenograft tumor formation. Huang et al. (2023) concluded that the mechanism of Pulsatilla decoction in treating GC may be related to the regulation of NFKBIA. Lin et al. (2019) reported that MT2A can target the promoter of NFKBIA by interacting with MZF1, and then play an anti-GC role. All of these results collectively suggest that NFKBIA may be an inhibitor of GC. In line with earlier studies, we discovered that shNFKBIA overrode the suppressive impact of miR-668-3p inhibitor on GC progression, suggesting that miR-668-3p could stimulate GC progression through its inhibition of NFKBIA.

One of the highlights of this study is that the miR-668-3p/NFKBIA axis can regulate the M2 polarization of macrophages. NFKBIA was previously identified by Li et al. (2021) as a macrophage marker gene, indicating that it might be crucial for deciphering the biological behavior of macrophages. Additionally, it was discovered that miR-668 is the target gene of circRNA-0003528, which influences the progression of tuberculosis by controlling macrophage polarization (Huang et al. 2020). Our research demonstrates that the miR-668-3p inhibitor may stimulate the M1 phenotype, which can be inhibited by shNFKBIA. This implies that miR-668-3p suppresses NFKBIA in GC cells to promote M2 polarization of THP-1 macrophage. It is worth noting that the crosstalk between macrophages and GC cells can induce the progression of malignant tumors (Piao et al. 2022). Exosomal miRNA secreted by GC has been reported to facilitate GC liver metastases (Qiu et al. 2022). By secreting the protein CHI3L1, M2 macrophages may foster the expansion of GC (Chen et al. 2017). In HER2-positive gastric cancer, M2 macrophages can also cause trastuzumab drug resistance (Hu et al. 2023). In line with prior research, we witnessed the progression of a malignant phenotype in GC cells co-cultured with THP-1 macrophages. This indicates that miR-668-3p may stimulate macrophage M2 polarization by suppressing NFKBIA in GC cells, which in turn stimulates the progression of GC.

But this study is not without its flaws. First off, there were no studies done on animals. Second, the way of communication—possibly via exosomes—between GC cells

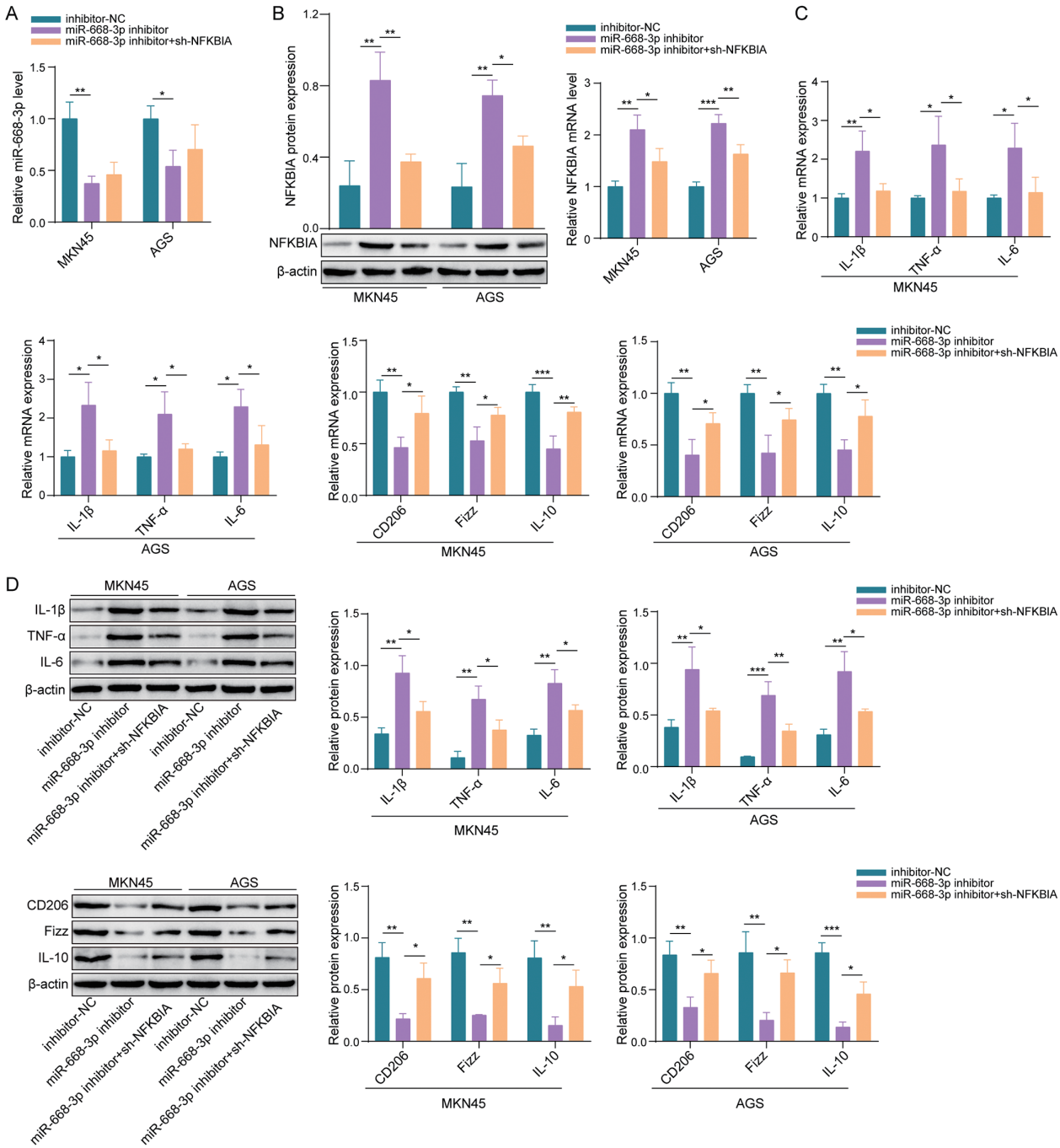

Fig. 3. miR-668-3p regulates M2 macrophage polarization by inhibiting NFKBIA.
The supernatants of transfected gastric cancer cells were co-cultured with THP-1 macrophages. (A) The expressions of miR-668-3p in THP-1 macrophage were determined by qPCR. (B) The expressions of NFKBIA in THP-1 macrophage were determined by qPCR or western blot. (C) The expressions of IL-1β, TNF-α, IL-6, CD206, Fizz1 and IL-10 were determined by qPCR. (D) The expressions of IL-1β, TNF-α, IL-6, CD206, Fizz1 and IL-10 were determined by western blot. Data were displayed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

and macrophages is unclear. To confirm all of these, more research is required.

In summary, our study elucidated the contribution of miR-668-3p/NFKBIA axis to the progression of GC, and its mechanism was specifically to promote M2 polarization of macrophages. This suggests that developing GC immunotherapy medicines that specifically target miR-668-3p and NFKBIA may be appealing.

Author Contributions

Chenghao Chu guaranteed the integrity of the entire study. Chenghao Chu and Bin Liu designed the study and literature research. Bin Liu defined the intellectual content. Yongwei Zhang and Zhangxuan Xu performed experiment

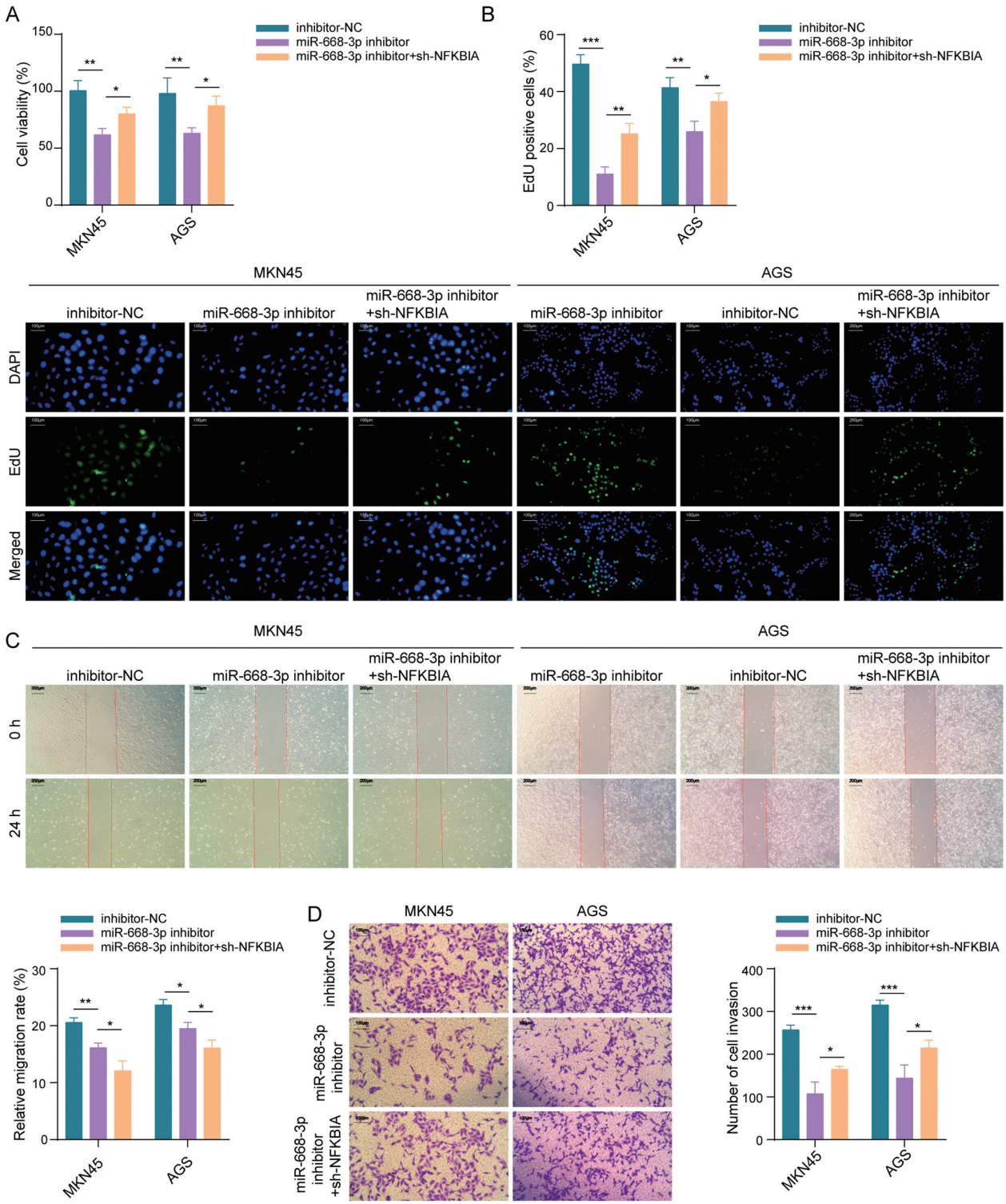

Fig. 4. miR-668-3p/NFKBIA axis regulates M2 macrophage polarization to facilitate proliferation, invasion and migration of gastric cancer cells.

Transfected gastric cancer cells were seeded in the lower chamber of Transwell, while the gastric cancer cells' supernatant treated THP-1 macrophages were seeded in the upper chamber of Transwell. (A) The viability of gastric cancer cells was determined by CCK-8. (B) The proliferation of gastric cancer cells was determined by EdU staining. (C) The migration of gastric cancer cells was determined by wound healing assay. (D) The invasion of gastric cancer cells was determined by transwell assay. Data were displayed as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

and collected the data. Bin Wang and Kai Ling Chin analyzed the data. Chenghao Chu and Bin Liu wrote the main manuscript and prepared Figs. All authors reviewed the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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