Long non-coding RNA (FALEC), as a lncRNA, Promotes Malignant Behaviors of Gastric Cancer Cells by Regulating miR-203b/PIM3 Axis

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Research Article

Keywords: Gastric cancer, focally amplified lncRNA on chromosome 1, microRNA-203b, PIM3, autophagy

Posted Date: October 5th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-942059/v1>

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Abstract

Background

Pieces of evidences have shown the important regulatory effects of long non-coding RNAs (lncRNAs) in gastric cancer (GC). While it is not entirely clear for the role and mechanism of focally amplified lncRNA on chromosome 1 (FALEC) during GC tumorigenesis.

Methods

The levels of FALEC, microRNA-203b (miR-203b), and PIM3 were confirmed by qRT-PCR. And cell autophagy, proliferation, apoptosis, migration, and invasion were estimated using western blot for autophagy-related proteins, Transmission electron microscopy (TEM), CCK-8, flow cytometer, and Transwell assays in NCI-N87 cells and Xenograft tumor. Besides, the interaction between miR-203b and FALEC or PIM3 was verified using a dual-luciferase reporter assay. Moreover, the involvement of miR-203b and PIM3 in the regulatory effects of FALEC on GC was determined by rescue experiments.

Results

The results proved that FALEC and PIM3 were highly expressed, while miR-203b was lowly expressed in GC. FALEC knockdown repressed GC cell proliferation, migration, and invasion, promoted apoptosis and autophagy *in vitro*. Meanwhile, FALEC knockdown prevented growth and induced GC autophagy *in vivo*. In mechanism, FALEC could upregulate PIM3 by sponging miR-203b in GC cells. Besides, FALEC induced the malignant behaviors of GC cells by regulating the miR-203b/PIM3 axis.

Conclusions

Therefore, FALEC/miR-203b/PIM3 axis might act as the promising therapeutic target for the therapy of GC patients.

Introduction

Gastric cancer (GC) is the most common gastrointestinal malignancy that originates from the lining of the stomach [1]. Although the incidence of GC has been largely reduced in the developed countries during the past decades due to the tremendous progress achieved in the diagnosis and therapy of GC, GC remains as a major threat to human health in the developing world [2]. It was reported that more than two hundred thousand GC-related deaths occurred in China each year, this number even over half of all GC-related deaths in the world [3]. The backward of cancer screening technologies resulting in a large portion of GC patients are diagnosed at advanced stages, which were featured by malignant proliferation, distant metastasis, and invasion, all of which are closely correlated with poor prognosis and high mortality [4]. Nowadays, gastrectomy remains to be the foremost therapeutic option for GC patients, however, it may be an impracticable option for those GC patients suffering from extensive invasion or lymphatic metastasis [5]. The five-year survival rate of advanced GC patients remains below 5% even after receiving

systemic therapy [6]. Thus, it would be of great importance that elucidation is supplied in regard to the mechanism by which GC tumorigenesis operates, to develop effective GC tumor markers in an attempt to improve the prognosis of GC patients.

Long non-coding RNAs (lncRNAs) is an important transcript with over 200 nucleotides [7]. Numerous studies have shown the critical role served by lncRNAs as important modulators in multiple cellular biological events involved with cancer, including proliferation, death, migration, and invasion [8]. In GC, some lncRNAs have been identified to be upregulated and act as oncogenes, while some lncRNAs exhibited downregulation and play as repressors [9]. FALEC has previously been revealed to affect the occurrence and progression of various human tumors, such as tongue squamous cell carcinoma, endometrial cancer, cervical cancer [10-12]. Recently, FALEC was reported to be upregulated in GC and its upregulation is closely correlated with lymph node metastasis and TNM stages [13]. Nevertheless, its functional roles and underlying mechanisms in GC remain largely unclear.

Autophagy is a highly conserved intracellular homeostatic pathway that is responsible for the degradation of damaged intracellular organelles and long-lived proteins [14]. Cells can obtain energy during the autophagic process under various cellular stresses, including nutrient-depleted, ischemia, oxidative stress [15, 16]. In tumor cells, autophagy may act as a self-defensive mechanism that contributes to tumor cell survival by removing toxins and garbage [17, 18]. However, abnormal autophagic activity may cause the inappropriate degradation of cell components that are indispensable for tumor cell survival, resulting in autophagic cell death [19]. Additionally, lncRNAs have been shown to have a key regulatory effect on tumor cell autophagy, and thus, involves in the tumorigenesis of GC [20].

In this study, we aimed to investigate the functional roles of FALEC in GC and attempted to figure out whether autophagy involves the functional role of FALEC in GC, as well as the potential molecular mechanisms.

Materials And Methods

Tissue collection and cell culture

Twenty pairs GC and adjacent para-carcinoma tissues were collected from patients who were diagnosed as GC blinded for peer review, and maintained under -80°C until use. This study was approved by the Ethics Committee of blinded for peer review. Informed consent was provided to each patient. GES-1 (gastric epithelial cell line) as well as AGS, MGC803, and NCI-N87 (GC cell lines) were all purchased from ATCC and maintained in an incubator with DMEM medium supplemented with 10% fetal bovine serum and 1% Penicillin&Streptomycin at 37°C.

Quantitative real-time PCR (RT-PCR) assay

Relative expression levels of FALEC, miR-203b, and PIM3 mRNA of GC tissues and cells were examined with qRT-PCR. The total RNAs were extracted after lysing in TRIzol reagent (Invitrogen, USA), followed by

the qualifying examination. Next, 3 µg of extracted RNAs were employed as a template to generate cDNA with the help of the Bestar™ qPCR RT kit (DBI Bioscience, China). RT-PCR process was conducted on a 7500 Fast Real-Time PCR system (Applied Biosystems, USA) using Power SYBR green master mix (Thermo Fisher Scientific, USA). FALEC and PIM3 expressions were normalized to β-actin, MiR-203b was normalized to U6. The gene expression was quantitated using the $2^{-\Delta\Delta C_q}$ method from 3 independent repetitions[21]. Sequences of primers used in the present study were listed in **Table 1**.

Transfection of oligonucleotide

Oligonucleotides including siRNAs against FALEC (si-FALEC), miR-203b mimics, FALEC, and PIM3, as well as their negative controls, were designed and obtained from Integrated Biotech Solutions (Shanghai, China). Oligonucleotide transfection was conducted using Lipofectamine 3000 (Invitrogen) following its protocol.

Western blot

Total proteins were isolated from GC cells after indicated treatment using RIPA buffer, and protein concentration was determined with the help of a BCA kit (Beyotime, China). Protein samples (50 µg) were separated in SDS-PAGE followed by transferring into PVDF membranes (Millipore, USA). Afterwards, membranes were blocked in 5% non-fat milk for 2 h and incubated with the primary antibodies for 8 h against LC3B (1:1000, ab48394, Abcam), Beclin 1 (1:2000, ab207612, Abcam), P62 (1:500, ab91526, Abcam), PIM3 (1:2000, ab154729, Abcam) and GAPDH (1:10000, ab181602, Abcam). HRP-conjugated donkey-anti-rabbit IgG secondary antibody (Abcam) was used to probe primary antibody for 2 h. Bands were visualized by the ECL system (Thermo Fisher Scientific) and analyzed by Image J.

Transmission electron microscopy (TEM)

TEM was used to monitor the autophagy status of treated GC cells. In brief, treated GC cells were collected and washed with PBS followed by fixation in 2% glutaraldehyde for 2 h. Cells were then washed with PBS three times and subjected to post-fixation in 1% osmium tetroxide for 1 h and dehydration in graded ethanol for 2 h. TEM observation was conducted with a Leo 912 AB electron microscope.

IF staining

Treated GC cells were fixed in 4% paraformaldehyde for 30 min and then incubated with TBS containing 5% Tween-20 for 2 h. After block in 10% normal goat serum for 1 h, IF staining was conducted using LC3 primary antibody (1:100, ab62720, Abcam) incubation overnight and subsequent secondary antibody incubation (DAR-546, Abcam) for 2 h. DAPI was used to label nuclear for 30 min. Images were photographed using a confocal laser microscope (LSM710, Zeiss, Germany).

Cell proliferation assessment

Cell proliferation was monitored by the CCK-8 kit (Lianke Bio, China). Briefly, cells were harvested after treatment at exponential proliferation phase and plated onto 96-well plates, followed by 1-, 2-, and 3-days incubation at 37°C. CCK-8 solution was added to each well and allowed to incubate for 10 min. The absorbance of each well was measured at 450 nm.

Cell apoptosis assessment

Cell death was estimated by a flow cytometer (BD Bioscience, USA) after staining with Annexin V-FITC Apoptosis Detection Kit (BioVision, USA) and PI following the protocols of manufacturers. Data was analyzed in CellQuest software.

Cell migration and invasion assessment

Cell migratory and invasive capacities were measured using transwell assay. In brief, transwell chambers (8 µm, Corning, USA) coated with or without Matrigel were adopted to evaluate the invasive or migratory ability, respectively. Treated GC cells (1×10^5 cells) suspended in 500 µL DMEM were added into the upper chamber, and 500 µL FBS-included DMEM was added into the lower one. After incubating at 37°C for 24 h, non-migrated or non-invaded cells were removed and those migrated or invaded through the membranes were fixed in 4% paraformaldehyde and stained with crystal violet. The number of migratory and invasive cells was counted manually under 10x time magnification.

Dual-luciferase reporter assay

To verify the interaction between miR-203b and FALEC or PIM3, a dual-luciferase reporter assay was carried out. The wild type and mutant miR-203b binding sequence of FALEC (FALEC-WT, FALEC-MUT) and PIM3 (PIM3-WT, PIM3-MUT) were amplified and inserted into pGL3 luciferase reporter vector to generate recombinant luciferase reporter plasmids. To verify the interaction between miR-203b and FALEC, miR-203b mimics or inhibitor was co-transfected into cells with FALEC-WT or FALEC-MUT, followed by the luciferase intensity assessment using a Dual-Luciferase Reporter Assay System (Promega). The verify of the interaction between miR-203b and PIM3 was carried out as same as described above.

Immunohistochemical (IHC) analysis

GC tumor tissues were cut into 5 µm slices after fixation in 4% paraformaldehyde overnight. Slices were incubated with 10% normal donkey serum and then incubated with anti-PIM3 (1:2000, ab154729, Abcam) primary antibody for 8 h. After washing with PBS, slides were incubated with HRP-conjugated secondary antibody for 1 h. Slices were photographed under a phase-contrast light microscope (Olympus, Japan).

Xenograft tumor proliferation assay

Nude mice (Male, eight weeks old, n = 5) obtained from the central laboratory were kept in a vivarium under 12 h/12h day/night circle with free access to food and water. Animal manipulations were approved by blinded for peer review. NCI-N87 cells stably expressed NC or si-FALEC were inoculated

subcutaneously into the mice and allowed to proliferate for four weeks. Tumors were dissected after four weeks of inoculation and used for further study. For Hematoxylin and Eosin (HE) staining, xenograft tumors were fixed in 4% paraformaldehyde and cut into μm slices, followed by HE staining using the H&E Staining Kit (ab245880, Abcam). RAB2B IHC analysis was carried out as described above.

Statistical Analysis

Data were presented as mean $\pm$ SEM. The student's t-test was employed to analyze the difference between groups using Graphpad (Ver. Prism 8, GraphPad Prism Software, USA). P-value less than 0.05 was considered significant.

Table 1. The sequences of primers in qRT-PCR assay

ID	Sequence (5'- 3')
β -actin	Forward: TGGATCAGCAAGCAGGAGTA
β -actin	Reverse: TCGGCCACATTGTGAACTTT
FALEC	Forward: AGGCAGCAGAACATACAGGA
FALEC	Reverse: CCGTTTGAAGTTGCTACCAC
MiR-203b	Forward: ACACTCCAGCTGGGTAGTGGTCCTAAACATTT
MiR-203b	Reverse: CTCAACTGGTGTCGTGGA
MiR-203b	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGTGAAAT
PIM3	Forward: AAGCAGTGACCTCTACCCCTGGTGACC
PIM3	Reverse: CAAATAAATTAAACAATAAATAGCCCC
U6	Forward: CTCGCTTCGGCAGCACA
U6	Reverse: AACGCTTCACGAATTTGCGT
U6	RT: AACGCTTCACGAATTTGCGT

Results

FALEC silencing repressed proliferation, migration, and invasion, and induced apoptosis and autophagy in GC cells

To investigate whether FALEC plays a role during tumorigenesis of GC, we firstly examined its expression in 20 pairs of GC and matched para-carcinoma tissues samples by qRT-PCR. As results indicated that FALEC was sharply upregulated in 19 GC tissues compared to para-carcinoma ones (Fig. 1A). Consistently, we found a remarkable upregulation of FALEC in GC cell lines AGS, MGC803, and NCI-N87 compared to that in GES-1 cells (Fig. 1B). Therefore, these findings suggested that FALEC may be

involved in the pathogenesis of GC. Besides, we estimated the effects of FALEC knockdown on GC cell functions. We first adopted qRT-PCR analysis to verify the silence effect of FALEC by siRNAs in NCI-N87 cells, and the results showed that FALEC expression was markedly reduced in the si-FALEC group relative to that in the si-NC group (Fig. 1C). Then we discovered that FALEC knockdown dramatically reduced NCI-N87 cell proliferation (Fig. 1D); FALEC knockdown notably increased the apoptosis rate of NCI-N87 cells (Fig. 1E). Additionally, we revealed that FALEC knockdown caused a prominent repressive effect on the migratory and invasive capacities of NCI-N87 cells (Fig. 1F and 1G). Also, compared to the si-NC group, Beclin 1 expression was markedly increased, while P62 expression was markedly decreased in The FALEC-silenced group (Fig. 1H). Moreover, results from TEM indicated a substantial increase in the accumulation of autophagic vesicles in si-FALEC transfected NCI-N87 cells (Fig. 1I). And a prominent upregulation of LC3B was also discovered in the si-FALEC group relative to the si-NC group (Fig. 1J). In consequence, our data indicated that FALEC knockdown promoted GC cell apoptosis and autophagy, while repressed GC cell proliferation, migration, and invasion *in vitro*.

MiR-203b was sponged by FALEC

Then we screened the potential target miRNAs of FALEC using the starBase database. Through qRT-PCR analysis, we discovered that miRNAs including miR-203b, miR-2116-3p, miR-619-5p, miR-1972, and miR-6875-5p were prominently downregulated in FALEC-silenced NCI-N87 cells, especially miR-203b (Fig. 2A). And miR-203b was selected for further study. Next, qRT-PCR analysis of miR-203b showed that it was remarkably downregulated in 20 GC tissues (Fig. 2B). Moreover, we found a negative correlation between the expression of FALEC and miR-203b in GC (Fig. 2C). Next, we verified the physical interaction between FALEC and miR-203b. The luciferase activity of GC cells driven by FALEC-WT was sharply attenuated by the transfection of miR-203b mimics (Fig. 2D). In summary, miR-203b can target FALEC in GC.

FALEC relieved the regulatory effects of miR-203b overexpression on GC cell functions

To determine whether miR-203b involves the regulatory effects of FALEC on GC progression, NCI-N87 cells were first transfected with miR-203b mimics and FALEC plasmid. As presented in Fig. 3A and 3B, transfection of miR-203b mimics could upregulate miR-203b, then overexpression of FALEC could memorably upregulate FALEC and downregulate miR-203b in miR-203b-overexpressed NCI-N87 cells. Subsequently, CCK-8 data revealed that compared to The NC group, cell proliferation in the miR-203b group was significantly repressed, while co-transfection of miR-203b and FALEC partially reversed the inhibitory effects of miR-203b mimics on cell proliferation (Fig. 3C). Results of apoptosis analysis showed that transfection of miR-203b mimics caused a conspicuous increase of cell apoptosis, while this phenomenon was partially abolished by FALEC (Fig. 3D). Moreover, the transwell assay indicated significant inhibitory effect on the migratory and invasive capacities in the miR-203b group, which also could be abrogated by FALEC (Fig. 3E and 3F). Meanwhile, we estimated the change of cell autophagy. PIM3 and P62 expressions were observably decreased, while Beclin 1 was notably upregulated in miR-203b-overexpressed NCI-N87 cells, while these dysregulations could be reversed by FALEC overexpression (Fig. 3G). A significant increase of autophagic vesicles was also observed in the miR-203b-overexpressed

NCI-N87 cells, while this increase could be abolished by FALEC overexpression (Fig. 3H). And the data indicated that miR-203b overexpression caused a prominent induction on autophagy (LC3B), while FALEC overexpression could abrogate this effect (Fig. 3I). As a consequence, FALEC reversed the inhibitory effects of miR-203b overexpression on GC cell functions *in vitro*.

PIM3 was targeted by miR-203b

By TargetScan, we found five genes including PIM3, CDKL3, ATP8A1, COLGALT2, and FUT8 might be the potential target genes of miR-203b. To screen for the most effective target genes, qRT-PCR was conducted in miR-203b-overexpressed NCI-N87 cells. And the results proved that overexpression of miR-203b could cause prominent downregulation of PIM3, CDKL3, ATP8A1, and COLGALT2 were in NCI-N87 cells, especially PIM3 (Fig. 4A). and PIM3 was selected and verified in subsequent experiments. Firstly, IHC data proved that PIM3 was upregulated in GC tissues (Fig. 4B). Secondly, PIM3 also indicated a prominent upregulation of PIM3 in GC tissues compared to para-carcinoma tissues (Fig. 4C and 4D). Meanwhile, we discovered that the silence of FALEC could memorably downregulate PIM3 in NCI-N87 cells (Fig. 4E). Besides, a positive correlation was observed between PIM3 and FALEC expression (Fig. 4F), and a negative correlation was determined between PIM3 and miR-203b expression (Fig. 4G). Physical interaction between miR-203b and PIM3 was also verified. Luciferase activity of GC cells driven by PIM3-WT was remarkably attenuated by miR-203b mimics and was remarkably enhanced by miR-203b inhibitor (Fig. 4H). Thus, these results suggested that PIM3 was targeted by and inversely correlated with miR-203b in GC.

FALEC overexpression induced proliferation, migration, and invasion, and prevented apoptosis and autophagy through the miR-203b/PIM3 axis

We further determined whether PIM3 participates in the regulatory effects of FALEC/miR203b on the malignant behaviors of GC. qRT-PCR results uncovered that the transfection of miR-203b mimics could notably downregulate FALEC and PIM3, while overexpression of PIM3 and FALEC could markedly reverse the levels of FALEC and PIM3 mediated by miR-203b overexpression in NCI-N87 cells (**Fig. 5A and 5C**). Meanwhile, we discovered that overexpressed-miR-203b, which was induced by miR-203b mimics, could be dramatically weakened by overexpression of FALEC in NCI-N87 cells (**Fig. 5B**). And the results of functional experiments displayed that the repressive effects of miR-203b overexpression on cell proliferation, migration, and invasion were reversed by PIM3 overexpression (**Fig. 5D, 5F-5G**). Similarly, our data also verified that the promotive effects of miR-203b overexpression on cell apoptosis also could be reversed by PIM3 overexpression (**Fig. 5E**). Moreover, the rescue effects of PIM3 also could be further enhanced by FALEC overexpression (**Fig. 5D-5H**). Next, the PIM3 overexpression could reverse the downregulation of PIM3 and P62 and the upregulation of Beclin1 induced by miR-203b overexpression, while this phenomenon could be blocked by FALEC overexpression (**Fig. 5H**). Then TEM results indicated that the stimulative effect of miR-203b overexpression on autophagosomes could be relieved by PIM3 overexpression, and this phenomenon also could be abolished by FALEC overexpression (**Fig. 5I**). This

conclusion was further supported by the IF staining of LC3B (**Fig. 5J**). Consequently, we revealed that FALEC could prominently accelerate GC progression through miR-203b/PIM3 axis.

FALEC knockdown inhibited growth and induced autophagy *in vivo*

To further determine the role of FALEC in GC tumor proliferation and autophagy *in vivo*, a xenograft tumor assay was performed. NCI-N87 cells were transfected with noting (blank), NC, and si-FALAEC, and the expression changes of FALEC, miR-203b, and PIM3 were identified through qRT-PCR assay. As displayed in **Fig. 6A**, silence of FALAEC markedly reduced FALEC and PIM3 expressions and elevated miR-203b expression in NCI-N87 cells ($P<0.001$). Next, FALAEC-silenced NCI-N87 cells were subcutaneously inoculated into the left flank of nude mice, and allowed to proliferation for four weeks (**Fig. 6B**). And by calculating, we discovered that the volume of tumors was remarkably smaller in the si-FALAEC group than in the NC group (**Fig. 6C**). Moreover, we detected the expression of FALEC, miR-203b, and PIM3 in the xenograft tumors formed by si-FALEC transfected cells by qRT-PCR. As results indicated that FALEC and PIM3 were observably downregulated while miR-203b was sharply upregulated in the si-FALEC transfected group compared to The NC group (**Fig. 6D-6F**). Next, H&E staining results indicated that the tumors formed by si-FALEC transfected cells showed well differentiation (**Fig. 6G**). Immunohistochemical analysis revealed a conspicuous downregulation of PIM3 in xenograft tumors formed by si-FALEC transfected cells (**Fig. 6H**). Results from western blot analysis indicated that PIM3 and P62 expression was markedly decreased, while Beclin 1 expression was markedly increased in the si-FALEC group (**Fig. 6I**), indicating that autophagy in the si-FALEC group was notably promoted. Meanwhile, the promotion of autophagy was further supported by the TEM results (**Fig. 6J**). These findings indicated that FALEC knockdown regulated autophagy, miR-203b, and PIM3 *in vivo*.

Discussion

FALEC, a 566 nucleotides lncRNA transcript locates at 1q21.2, has been reported to be an important oncogene in multiple human cancers, including prostate cancer, papillary thyroid carcinoma, and melanoma [22-24]. However, it was revealed to be remarkably downregulated in tongue squamous cell carcinoma (TSCC), and exhibited a repressive effect on TSCC cell proliferation and metastasis by silencing extracellular matrix protein 1 (ECM1) through EZH2 [10]. Thus, its expression level and biological function in different types of cancer may be different. FALEC was revealed, recently, to be increased in GC, and knockdown of FALEC was found to inhibit GC progression by impairing ECM1 expression [13]. Consistently, in our study, we confirmed the upregulation of FALEC in GC tissues and cell lines, and knockdown of FALEC exhibited a remarkable repressive effect on GC progression *in vitro* and *in vivo*. Moreover, we found that FALEC knockdown could induce GC cell autophagy. And in mechanism, FALEC could regulate the expression of PIM3 by sponging miR-203b in GC and thus regulating the autophagy, proliferation, apoptosis, migration, and invasion of GC cells.

It is generally known that lncRNAs share a general functionality in their capacity to regulate gene expression by sponging miRNAs in a phenomenon defined as competing for endogenous RNA (ceRNA)

theory [25, 26]. The hypothesis of ceRNA has been demonstrated to be a common mechanism of lncRNA in regulating tumor progression [27]. MiRNAs have functions similar to oncogenes or tumor suppressor genes and are closely related to the development process of human tumors[28]. Recent studies have uncovered that miRNAs are relevant to the occurrence, development, and metastasis of GC[29, 30]. Thus, we screened the target miRNAs of FALEC by the starBase database. The new studies confirmed that miR-203b could act as a tumor inhibitor in a variety of cancers, including Gastric Cardia Adenocarcinoma[31], esophageal squamous cell carcinoma[32], skincancer[33], and colorectal cancer[34]. After screening, miR-203b was identified as the most promising one. We further discovered that miR-203b was strongly decreased in GC. Meanwhile, miR-203b overexpression could prevent proliferation, migration, and invasion, and induce apoptosis and autophagy, which also could be abolished by the overexpression of FALEC in GC cells. Besides, we also verified the physical interaction between miR-203b and FALEC. Therefore, we proved that FALEC accelerated the malignant biological properties of GC cells by targeting miR-203b.

Researchers have also proved that miRNAs can directly degrade mRNA or prevent translation negatively regulate target genes, thus affecting a number of cell activities, such as cell growth, proliferation, apoptosis, autophagy, inflammation, invasion, and metastasis[35, 36]. Additionally, we also predicted the potential target genes of miR-203b using TargetScan, and PIM3 was identified to be the potential target gene of miR-203b. PIM kinase family is a group of calcium/calmodulin regulated kinase (CAMP) families, whose members (PIM-1, PIM-2, PIM-3) are highly homologous in the kinase domain[37]. Researchers have testified that PIM family kinases can significantly induce cell survival and proliferation[38, 39]. Recent studies have indicated that PIM-3 is abnormally expressed in a variety of cancers, including prostate cancer[40], colorectal cancer[41], pancreatic cancer[42], rectal cancer[43], melanoma[44]. Therefore, PIM-3 might be a novel target for anti-tumor therapy. Nevertheless, the role of miR-203b and PIM3 in GC tumorigenesis has not been reported yet. In our study, we disclosed that PIM3 was a targeted gene of miR-203b, and could be negatively regulated by miR-203b in GC. also, we revealed that miR-203b/PIM3 axis could be involved in the regulation of FALEC on the autophagy, proliferation, migration, invasion, and apoptosis in GC. In summary, FALEC/miR-203b/PIM3 works as an axis to regulate the malignant activities of GC cells.

Tumor distant metastasis usually indicates advanced progression and poor overall survival rate of GC [45]. The tumor metastasis process is extremely complex, involving multiple pathological activities, including angiogenesis, autophagy, and epithelial-to-mesenchymal transition [46]. And autophagy is a highly conserved metabolic process involving multiple steps, which can maintain the homeostasis of the intracellular environment by degrading damaged proteins, cellular metabolites, and diseased organelles. LC3 and P62, as the key markers of autophagy, are frequently applied to evaluate autophagy levels. Beclin-1, as a tumor suppressor gene, is affected by autophagy during cancer progression. Moreover, induction of autophagy has been confirmed to be associated with the prevention of GC progression in multiple studies[47, 48], which is consistent with our conclusion.

In conclusion, the results from the *in vitro* and *in vivo* experiments suggested that FALEC accelerates GC cell malignant behaviors by regulating the miR-203b/PIM3 axis, which might provide a potential therapeutic axis for GC treatment.

Declarations

Ethics approval and consent to participate

All protocols followed the requirements of the Animal Experiment Center of the Institute.

Consent for publication

Not applicable.

Availability of data and material

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors have no commercial or other associations that might pose a conflict of interest.

Funding

Not applicable.

Authors' contributions

SW and WD participated in the study design, and manuscript writing. SW, JX and WD participated in the image data acquisition. WD and HL participated in statistical analysis. WD, MG, JX, HL, MT and SW participated in the experimental operation. All authors read and approved the final manuscript.

Acknowledgments

Not applicable.

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Figures

Figure 1

FALEC silencing repressed GC cell proliferation, migration, and invasion, and induced apoptosis and autophagy. (A) Relative expression of FALEC was monitored by qRT-PCR in 20 pairs of GC tissues and matched para-carcinoma tissues. (B) FALEC expression was also confirmed by qRT-PCR in normal human

gastric epithelial cell line GES-1 and GC cell lines (AGS, MGC803, and NCI-N87). (C) The silencing effect of FALEC by siRNAs was determined using qRT-PCR analysis in NCI-N87 cells. After 24 h of transfection with nothing, si-NC or si-FALEC, NCI-N87 cells were subjected for proliferation and apoptosis assessments by CCK-8 (D) and flow cytometry analysis (E), respectively. (F-G) Migratory and invasive capacities were evaluated by transwell assay. (H) The protein expression levels of Beclin 1 and P62 were measured using western blot. (I) TEM was adopted to assess autophagy. (J) IF analysis of LC3B expression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2

MiR-203b was sponged by FALEC and inversely correlated with FALEC in GC. (A) After transfection with si-FALEC, the levels of the predicted miRNAs, which have potential binding sites with FALEC, were certified by qRT-PCR in NCI-N87 cells. (B) qRT-PCR analysis of miR-203b in 20 pairs GC tissues and matched para-carcinoma tissues. (C) Correlation between the expression of FALEC and miR-203b in GC tissues. (D) Interaction between FALEC and miR-203b was verified by dual-luciferase reporter assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3

FALEC relieved the regulatory effects of miR-203b overexpression on GC cell proliferation, apoptosis, migration, invasion, and autophagy. NCI-N87 cells were transfected with miR-203b mimics and miR-203b plus FALEC-overexpressed plasmid, respectively. The expression changes of FALEC (A) and miR-203b (B) were identified through qRT-PCR analysis in the transfected NCI-N87 cells. (C and D) After 24 h of transfection with miR-203b mimics alone or plus FALEC-overexpressed plasmid, NCI-N87 cells were subjected for the proliferation and apoptosis assessments by CCK-8 and flow cytometry. (E-F) The migration and invasion were assessed by Transwell assay. (G) The levels of PIM3, Beclin 1, and P62 proteins were identified by western blot. (H) TEM was adopted to assess autophagy. (I) IF analysis of LC3B expression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. Mimics group.

Figure 4

PIM3 was targeted by miR-203b and inversely correlated with miR-203b in GC. (A) The levels of the potential target genes were evaluated by qRT-PCR in miR-203b-overexpressed NCI-N87 cells. (B) Immunohistochemical analysis of PIM3 in the GC tissues. (C) Western blot analysis of PIM3 in GC tissues. (D) qRT-PCR analysis of PIM3 in 20 pairs of GC and matched para-carcinoma tissue samples. (E) The effect of FALEC silencing on PI3M expression was determined by western blotting in NCI-N87 cells.

Correlation between the expression of PIM3 and FALEC (F) or miR-203b (G). (H) Physical interaction between miR-203b and PIM3 was tested by dual-luciferase reporter assay. *P<0.05, **P<0.01, ***P<0.001.

Figure 5

FALEC overexpression prevented GC progression through miR-203b/PIM3 axis. NCI-N87 cells were transfected with miR-203b mimics, miR-203b mimics+PIM3-overexpressed plasmid, and miR-203b mimics+PIM3-overexpressed plasmid+FALEC-overexpressed plasmid. The expression changes of FALEC (A), miR-203b (B), and PIM3 (C) were confirmed by applying qRT-PCR analysis in the transfected NCI-N87 cells. (D) CCK-8 and (E) flow cytometry were carried out to measure cell proliferation and apoptosis, respectively. (F-G) Transwell assay was performed to estimate the migratory and invasive abilities of treated NCI-N87 cells. (H) PIM3 and autophagy proteins were examined by western blot. (I) Autophagy status was estimated by TEM. (J) LC3B expression was tested by IF staining. *P<0.05, **P<0.01,

***P<0.001 vs. NC group; #P<0.05, ##P<0.01, ###P<0.001 vs. Mimics+vector group; &P<0.05, &&P<0.01, &&&P<0.001 vs. Mimics+PIM3+vector group.

Figure 6

The role of FALEC knockdown in vivo. (A) FALEC, miR-203b, and PIM3 expressions were monitored by applying qRT-PCR assay in FALEC-silenced NCI-N87 cells. (B) Nude mice were injected subcutaneously with FALEC-silenced NCI-N87 cells. After 4 weeks, representative images of tumors were showed. (C) The size of the tumor was assessed by calculating the volume at the end of four weeks. Relative expression of FALEC (D), miR-203b (E), and PIM3 (F) were detected by qRT-PCR in xenograft tumors. (G) H&E staining and (H) anti-PIM3 immunostaining of xenograft tumors formed by FALEC silenced GC cells. (I) PIM3 and autophagy proteins were confirmed by western blot in the respective xenograft tumors. (J) The autophagy status of xenograft tumors was estimated by TEM. *P<0.05, **P<0.01, ***P<0.001.