

# MiR-195-5p suppresses gastric adenocarcinoma cell progression via targeting OTX1

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**Summary.** Gastric adenocarcinoma (GAC) caused by malignant transformation of gastric adenocytes is a malignancy with high incidence. MiR-195-5p modulates a variety of cancers. One of its target genes, orthodenticle homeobox 1 (OTX1), is believed to be a key modulator of tumor progression. We aim to analyze the mechanism of miR-195-5p and OTX1 in GAC. MiR-195-5p and OTX1 mRNA levels in GAC cells were tested via qRT-PCR. OTX1 protein and EMT-related protein levels were examined through western blot. Several cell functional assays were designed to measure changes in cell malignant behaviors. Dual luciferase assay verified the targeting relation of miR-195-5p and OTX1. These experimental results showed significantly low miR-195-5p expression and significantly high OTX1 expression in GAC cells. Enforced miR-195-5p level repressed cell malignant progression and accelerated cell apoptosis in GAC. Increased OTX1 weakened the above-mentioned effect caused by overexpressing miR-195-5p. Thus, miR-195-5p restrained migration, proliferation, invasion and epithelial-mesenchymal transition process of GAC cells, and promoted cell apoptosis through regulating OTX1. A new insight is provided for searching for biomarkers or therapeutic targets of GAC.

**Key words:** miR-195-5p, Gastric adenocarcinoma, OTX1, Invasion, Migration, Proliferation, Epithelial-mesenchymal transition

## Introduction

Gastric cancer (GC) is a malignancy that ranks third in leading to cancer-associated deaths all over the world (Siegel et al., 2017). Gastric adenocarcinoma (GAC)

accounts for 95% of all GC cases (Curea et al., 2017). Though surgery, radiotherapy, chemotherapy and other methods are used for cancer treatment (Sitarz et al., 2018), inconspicuous early symptoms of GAC and delayed diagnosis of most patients result in poor treatment efficacy (Kemi et al., 2019). Finding novel approaches for GAC diagnosis and treatment, therefore, is of great meaning for early diagnosis and prognosis improvement.

MicroRNAs (miRNAs) modulate downstream gene levels, which have received extensive attention in recent years (Lagos-Quintana et al., 2001; Ambros, 2003). MiRNAs were initially considered to be important mechanisms for regulating physiological processes such as development and stress response, but cumulative evidence manifests that miRNAs affect many pathological processes as well (Leung and Sharp, 2010). An example is that miR-107 and miR-25 are overexpressed in GAC tissue, and can simultaneously target *LATS2*, thereby affecting proliferation, invasion and cell cycle of cancer cells (Usluogullari et al., 2015). A study (Tang et al., 2020) showed that miR-665 is up-regulated in GAC, which facilitates GAC cell malignant phenotypes. MiR-195-5p has also been extensively studied in varying cancers, including colorectal cancer (Bai et al., 2020), oral squamous cell carcinoma (Wang et al., 2017), etc., but few studies focus on its role in GAC. The unclear mechanism of miR-195-5p in GAC needs to be illustrated.

Orthodenticle Homeobox 1 (OTX1) belongs to the OTX family of homologous box (HB) genes (Klein and Li, 1999). OTX1 is vital in brain regionalization (Omodei et al., 2009), sensory organ development (Acampora et al., 2000), and early human embryo retina (Larsen et al., 2009) and mammary gland development (Pagani et al., 2010) during embryonic development. What's more, recent studies found that OTX1 was also aberrantly expressed in colorectal cancer (Li et al., 2020), GC (Qin et al., 2018), medulloblastoma (de Haas et al., 2006) and other tumors, thus affecting the development of cancer cells. OTX1 is suggested to be a key regulator of tumor progression. Nonetheless, the

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function of the miR-195-5p/OTX1 axis in GAC still needs to be explored.

Here, we investigated roles and molecular mechanisms of miR-195-5p and OTX1 in GAC through a series of *in vitro* experiments. Our finding offers a foundation for developing new targets of GAC.

## Materials and methods

### Bioinformatics methods

From The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>), mature miRNA expression data and mRNA sequencing data of GAC (TCGA-STAD) were downloaded. After screening, miRNA data (cancer: 444; normal: 45) and mRNA data (cancer: 373; normal: 32) were obtained. MiR-195-5p expression was analyzed on the basis of downloaded data. R package edgeR was used to identify differential mRNAs ( $|\log_{2}FC| > 2$ ,  $padj < 0.05$ ). After that, the TargetScan ([http://www.targetscan.org/vert\\_72/](http://www.targetscan.org/vert_72/)), miRDB, (<http://mirdb.org/>), starBase, miRWalk (<http://mirwalk.umm.uni-Heidelberg.DE/>), mirDIP (<http://ophid.utoronto.ca/mirDIP>) databases were used for target gene prediction. Predicted results were overlapped with differential mRNAs. MiRNA-mRNA regulatory pair was determined by calculating correlation between miRNA and mRNA.

### Culture and transfection of cells

The cell line information is illustrated in Table 1. Incubation of all cell lines was done by mediums (Gibco, USA) plus 10% FBS. Culture medium types are shown in Table 1. Culture conditions were 37°C and 5% CO<sub>2</sub>. Lipofectamine 3000 kit (Invitrogen, USA) was introduced for cell transfection. We purchased miR-195-5p mimic (miR-mimic), NC mimic (miR-NC), si-NC, si-OTX1, pcDNA3.1-OTX1 plasmid (oe-OTX1) and pcDNA3.1 plasmid (oe-NC) encoding OTX1 from GenePharma (China). After rinsing the cells with PBS (pH 7.4), they were transfected with target plasmids and maintained in mediums with 5% CO<sub>2</sub> at 37°C for stand-

by use.

### qRT-PCR

Total RNA was separated with Trizol (Invitrogen, USA). In order to detect miR-195-5p expression, cDNA reverse transcription kit (Roche, Switzerland) and miR-195-5p specific reverse transcription primers (Biomics Biotech, China) were utilized to perform reverse transcription. SYBR Green PCR Master kit (Roche, Switzerland) and miR-195-5p qPCR were used to detect primer sets (Biomics Biotech, China). SYBR<sup>®</sup> Premix Ex Taq TM II (Takara, Japan) was utilized to measure the mRNA level of OTX1. The internal references U6 of miR-195-5p and GAPDH of OTX1 mRNA were assessed with SYBR Green PCR Master Kit (Roche, Switzerland). The qPCR temperature cycle was done as follows: 50°C for 2 min and 95°C for 10 min. Then 45 cycles were completed for 15 s at 95°C and 1 min at 60°C. Data were processed by Applied Biosystems 7500 real-time PCR system. Relative gene level was calculated by 2<sup>-ΔΔCt</sup> method. Table 2 exhibits primer sequences.

### MTT assay

The transfected GAC cell lines SGC-7901 and BGC-823 (5×10<sup>3</sup> cells/well) were digested, suspended, and then placed into 96-well plates. After 0, 24, 48 and 72h, each well was filled with 10 μL MTT reagent (5 mg/mL, Sigma-Aldrich, USA). After 4h of continuous culture at 37°C, supernatant was discarded, and precipitate was dissolved in 200 μL DMSO. The absorbance was measured at 595 nm at designated time points.

### Cell scratch assay

SGC-7901 and BGC-823 cells were plated in 6-well plates at 37°C. When cell coverage reached 90%, the tip of a 200 μL sterile pipette was gently scraped through the monolayer in the well center, and isolated cells were briefly rinsed twice to remove detached cells. DMEM plus 10% FBS was added, and cells grew again for 24h.

**Table 1.** Cell lines and culture mediums used in the experiment.

Type	Name	No.	Culture medium	Source
Human gastric mucosa cells	GES-1	HTX-1964	CM2-1 culture medium: 90% RPMI-1640+10% FBS. RPMI-1640: 1640 culture medium, containing glutamine	Otwo Biotech Inc. (Guangzhou, China)
Gastric adenocarcinoma cells	SGC-7901	HTX-2345	CM2-1 culture medium: 90% RPMI-1640+10% FBS. RPMI-1640: 1640 culture medium, containing glutamine	Otwo Biotech Inc. (Guangzhou, China)
	BGC823	HTX-1804	CM2-1 culture medium: 90% RPMI-1640+10% FBS. RPMI-1640: 1640 culture medium, containing glutamine	Otwo Biotech Inc. (Guangzhou, China)
	MKN45	HTX-2369	CM2-1 culture medium: 90% RPMI-1640+10% FBS. RPMI-1640: 1640 culture medium, containing glutamine	Otwo Biotech Inc. (Guangzhou, China)
	AGS	HTX-1739	CM7-1 culture medium: 90% F-12K+10% FBS. F-12K: F-12K culture medium.	(Guangzhou, China)

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A microscope was adopted to observe cell migration at 0 and 24h.

### Cell invasion assay

Transwell analysis was carried out in a 12-well Transwell chamber with 8- $\mu$ m aperture (Corning Costar, USA). Matrigel (50  $\mu$ l/well; BD Biosciences, USA) was supplemented to the upper chamber. After 2-3h at 37°C, cells ( $1 \times 10^5$  cells/mL) were plated into serum-free medium in the upper chamber. DMEM plus 10% FBS was filled into the lower chamber. Following 24h culture at 37°C, cells in the upper chamber were removed, while cells that passed through membrane were fixed in methanol, and dyed with crystal violet (0.04% in water). 4 random visual fields were observed under microscope to count invasive cells.

### Western blot

Radioimmunoprecipitation analysis buffer (Sigma-Aldrich, USA) was utilized to lyse SGC-7901 and BGC-823 cells after transfection, and total protein was measured by bicinchoninic acid method (Sigma-Aldrich, USA). After being isolated with 10% SDS-PAGE, samples were transferred onto a PVDF (Sigma-Aldrich, USA) membrane. Samples were blocked for 1 h at room temperature with 5% skim milk and maintained overnight with primary antibodies (rabbit anti-OTX1, E-cadherin, Snail, Vimentin and GAPDH) bought from Abcam (UK). Later, secondary antibody goat anti-rabbit IgG H&L (HRP) (Abcam, UK) was incubated with membrane for 1 h at room temperature. ECL reagent (SolarBio, China) was added for development, and gel imaging software was applied. The experiment was done 3 times, followed by statistical analysis.

### Dual-luciferase assay

OTX1 sequence containing miR-195-5p and mutated binding sites was amplified by PCR, followed by being cloned into pMIR vector downstream of Renilla luciferase reporter gene (Promega, USA). The wild-type OTX1 reporter vector (OTX1-WT) and the mutant-type

OTX1 reporter vector (OTX1-MUT) were established. SGC-7901 cells were co-transfected with OTX1-WT or OTX1-MUT and miR-195-5p mimic or NC mimic. The medium was changed 6 h later. We used a dual-luciferase reporter assay system (Promega, USA) and a Veritas microplate photometer (Turner Biosystems, USA) to assess luciferase activity.

### Cell apoptosis assay

Annexin V FITC Apoptosis Detection Kit I (Keygen Biotech, China) was utilized to detect cell apoptosis. GAC cell lines SGC-7901 and BGC-823 at logarithmic growth phase were treated with trypsin without EDTA, rinsed twice with PBS buffer, and then maintained at room temperature and in the dark for 15 min with 5  $\mu$ L/well Annexin-V-FITC and 5  $\mu$ L/well PI mixture. After incubation, cells were rinsed with 3 $\times$ PBS and suspended with 400  $\mu$ L binding buffer. Cell apoptosis was detected by flow cytometry (Thermo Fisher, USA).

### Analysis of statistics

All experiments underwent 3 independent repetitions. The data were handled on GraphPad Prism 6.0 (USA), and the results were presented as mean  $\pm$  SD. Spearman correlation was applied for analysis of correlation between miR-195-5p and OTX1. Comparison between two groups was done by *t*-test, and *p*<0.05 represented prominent difference.

## Results

### Significant low miR-195-5p expression in GAC

Differential analysis indicated markedly low miR-195-5p level in GAC (Fig. 1A). MiR-195-5p is less expressed in colorectal cancer as well (Lin et al., 2019). Since the mechanism of miR-195-5p affecting development of GAC cells has not been reported yet, miR-195-5p was the research of interest. Next, miR-195-5p level in GAC cells was dramatically down-regulated relevant to that in gastric mucosal cell line GES-1, as indicated by qRT-PCR detection result (Fig. 1B). In conclusion, a significant decrease of miR-195-5p was seen at tissue and cell levels in GAC. To further understand the mechanism of miR-195-5p, SGC-7901 and BGC-823 cells with marked difference in miR-195-5p level were selected.

### Forced miR-195-5p expression suppresses development of GAC cells

MiR-NC group and miR-mimic group were constructed based on transfection of SGC-7901 and BGC-823 cells. qRT-PCR was completed to assess transfection efficiency. Increased miR-195-5p level in the miR-mimic group (Fig. 2A) indicated that transfection efficiency met the requirements. MTT assay

**Table 2.** Primer sequences for qRT-PCR.

Gene	Primer sequence (5'→3')
miR-195-5p	F: GAATTCGCCTCAAGAGAACAAAGTGGAG R: AGATCTCCCATGGGGCTCAGCCCCCT
U6	F: AACGAGACGACGACAGAC R: GCAAATTCGTGAAGCGTTCATA
OTX1	F: CTGCTCTCCTCAATCAATGG R: ACCCTGACTTGTCTGTTTCC
GAPDH	F: TGCACCACCAACTGCTTAGC R: GGCATGGACTGTGGTCATGAG

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result displayed that cancer cell proliferation was remarkably constrained via overexpressing miR-195-5p (Fig. 2B). Next, Transwell and cell scratch assay results suggested that invasion and migration of GAC cells after overexpressing miR-195-5p were significantly restrained (Fig. 2C,D). Flow cytometry result exhibited that forced expression of miR-195-5p notably increased cell apoptosis level (Fig. 2E). Then, western blot result displayed that increased miR-195-5p level in SGC-7901 and BGC-823 cells notably enhanced epithelial marker E-cadherin level, but decreased levels of mesenchymal markers Snail and Vimentin (Fig. 2F). Therefore, forced miR-195-5p expression constrained malignant progression and EMT process of GAC cells, and promoted cell apoptosis.

### OTX1 is targeted and inhibited by miR-195-5p in GAC cells

To probe the downstream regulatory mechanism of miR-195-5p in GAC cells, we first conducted differential analysis on the mRNA data, and obtained totally 1662 differentially expressed mRNAs (775 up-regulated and 887 down-regulated) (Fig. 3A). Then, miR-195-5p targets were predicted with public databases. The intersection of predicted mRNAs and upregulated mRNAs was used to identify the 5 target genes (Fig. 3B). Correlation analysis of these 5 target genes and miR-195-5p showed that OTX1 had the strongest negative relation with miR-195-5p (Fig. 3C), and OTX1 was significantly up-regulated in cancer tissue (Fig. 3D). Next, we verified the modulatory impact of miR-195-5p on OTX1. TargetScan was utilized to predict binding sites of miR-195-5p and OTX1 3'-UTR (Fig. 3E). Afterward, the targeting relationship between them was validated through dual luciferase assay. It was shown that forced expression of miR-195-5p restrained luciferase activity of the OTX1-WT group, without having an impact on the OTX1-MUT group (Fig. 3F).

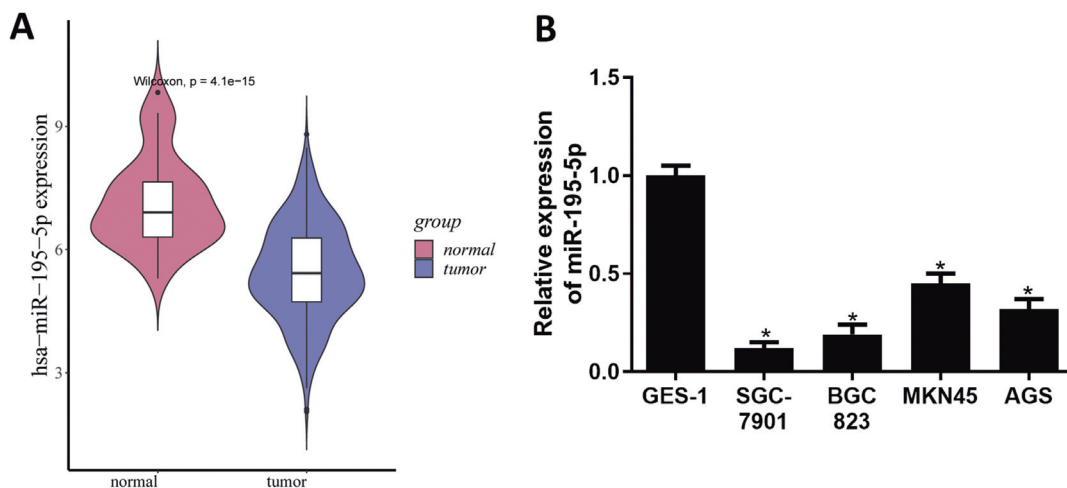
OTX1 mRNA and protein levels in varying transfection groups of SGC-7901 and BGC-823 cells were detected via qRT-PCR and western blot, which were conspicuously reduced in the miR-mimic group (Fig. 3G,H). Thus, miR-195-5p restrained OTX1 level in GAC cells.

### Loss of OTX1 expression inhibits the development of GAC cells

In view of the fact that OTX1 was up-regulated in GAC, we probed into the impact of OTX1 on progression of GAC cells by transfecting specific si-OTX1 into SGC-7901 and BGC-823 cells. qRT-PCR and western blot were introduced to verify knockdown efficiency of si-OTX1, and the result suggested that mRNA and protein levels of OTX1 in the si-OTX1 group were prominently decreased compared to those in control group (Fig. 4A,B). MTT assay result exhibited that inhibition of OTX1 expression significantly hampered the proliferation of GAC cells (Fig. 4C). Transwell assay and scratch assay revealed that invasion and migration of SGC-7901 and BGC-823 cells were hampered when OTX1 expression was down-regulated (Fig. 4D,E). Flow cytometry result showed that down-regulation of OTX1 dramatically elevated the apoptosis level of GAC cells (Fig. 4F). In conclusion, down-regulation of OTX1 inhibited malignant behaviors of GAC cells and stimulated cell apoptosis.

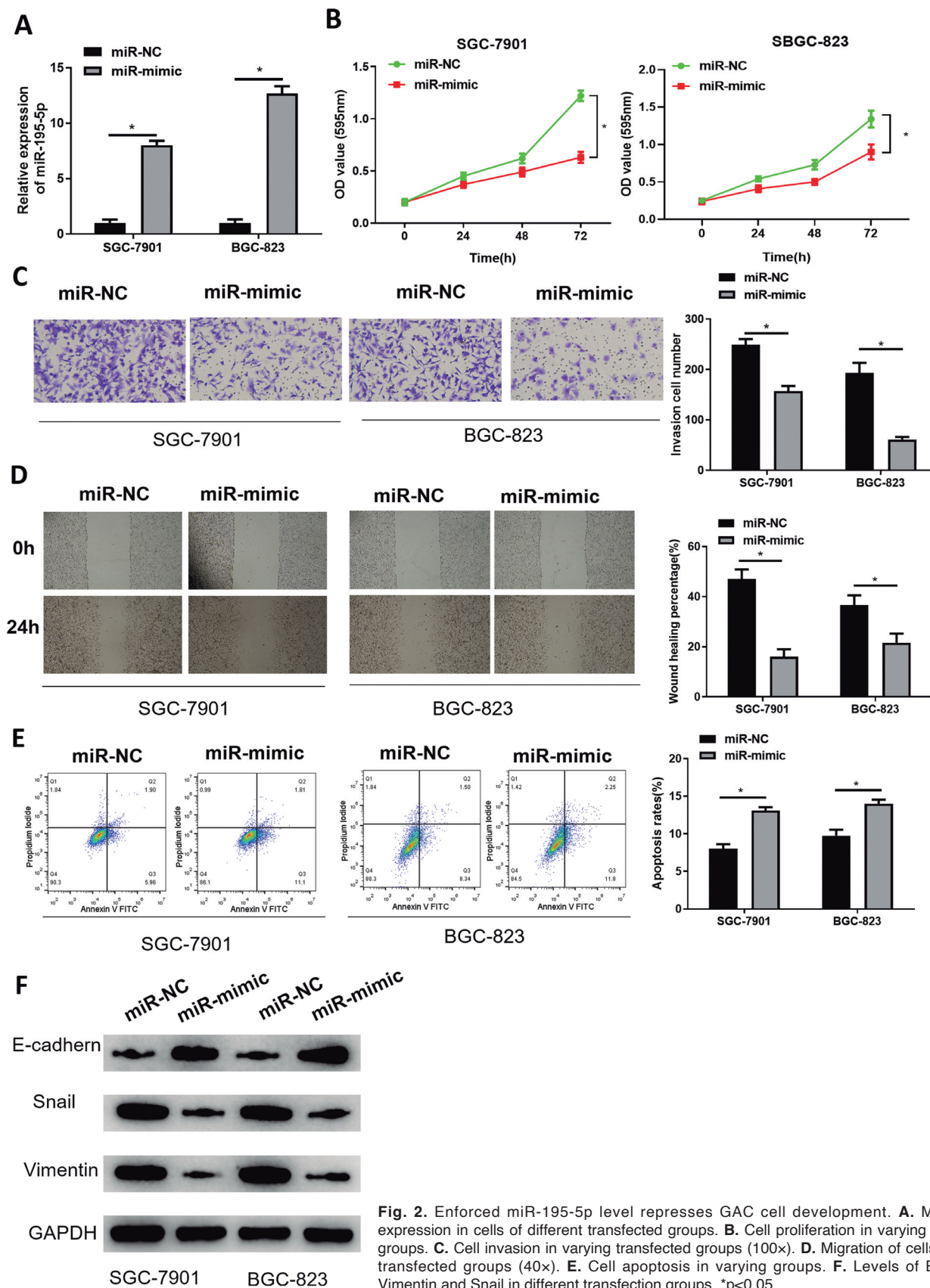
### MiR-195-5p restrains GAC cell development through targeting OTX1

To verify modulation of miR-195-5p and OTX1 in GAC cells, we constructed cell line overexpressing miR-195-5p and cell line concomitantly overexpressing miR-195-5p and OTX1. OTX1 mRNA and protein levels were examined via qRT-PCR and western blot. The experimental results presented that overexpression of



**Fig. 1.** MiR-195-5p expression is significantly low in GAC. **A.** The expression violinplot of miR-195-5p in the normal group (red) and the tumor group (blue). **B.** The expression of miR-195-5p in normal and GAC cells. \* $p < 0.05$ .

*MiR-195-5p/OTX1 axis restrained the development of GAC*

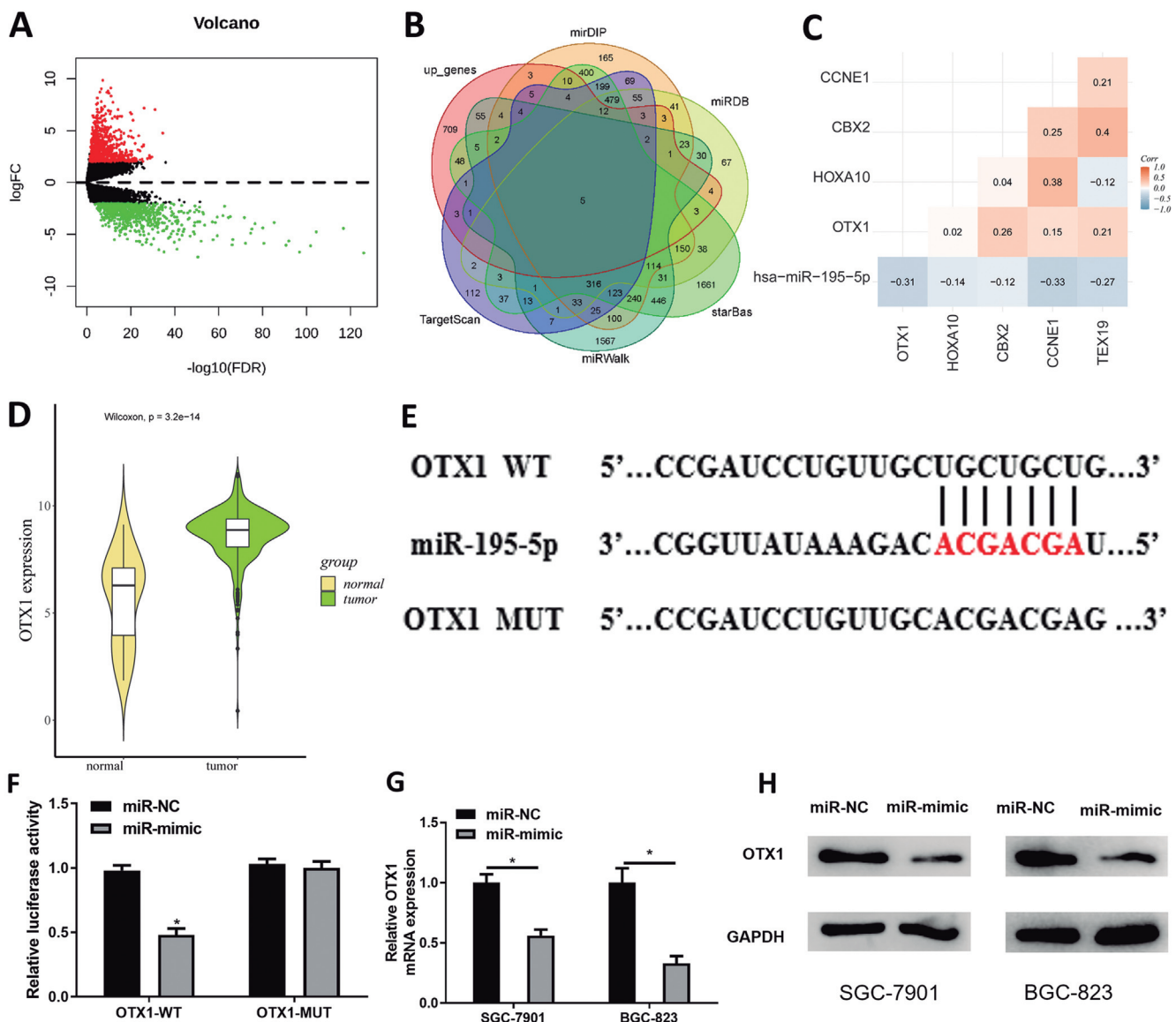


**Fig. 2.** Enforced miR-195-5p level represses GAC cell development. **A.** MiR-195-5p expression in cells of different transfected groups. **B.** Cell proliferation in varying transfected groups. **C.** Cell invasion in varying transfected groups (100 $\times$ ). **D.** Migration of cells in varying transfected groups (40 $\times$ ). **E.** Cell apoptosis in varying groups. **F.** Levels of E-cadherin, Vimentin and Snail in different transfection groups. \* $p < 0.05$ .

*MiR-195-5p/OTX1 axis restrained the development of GAC*

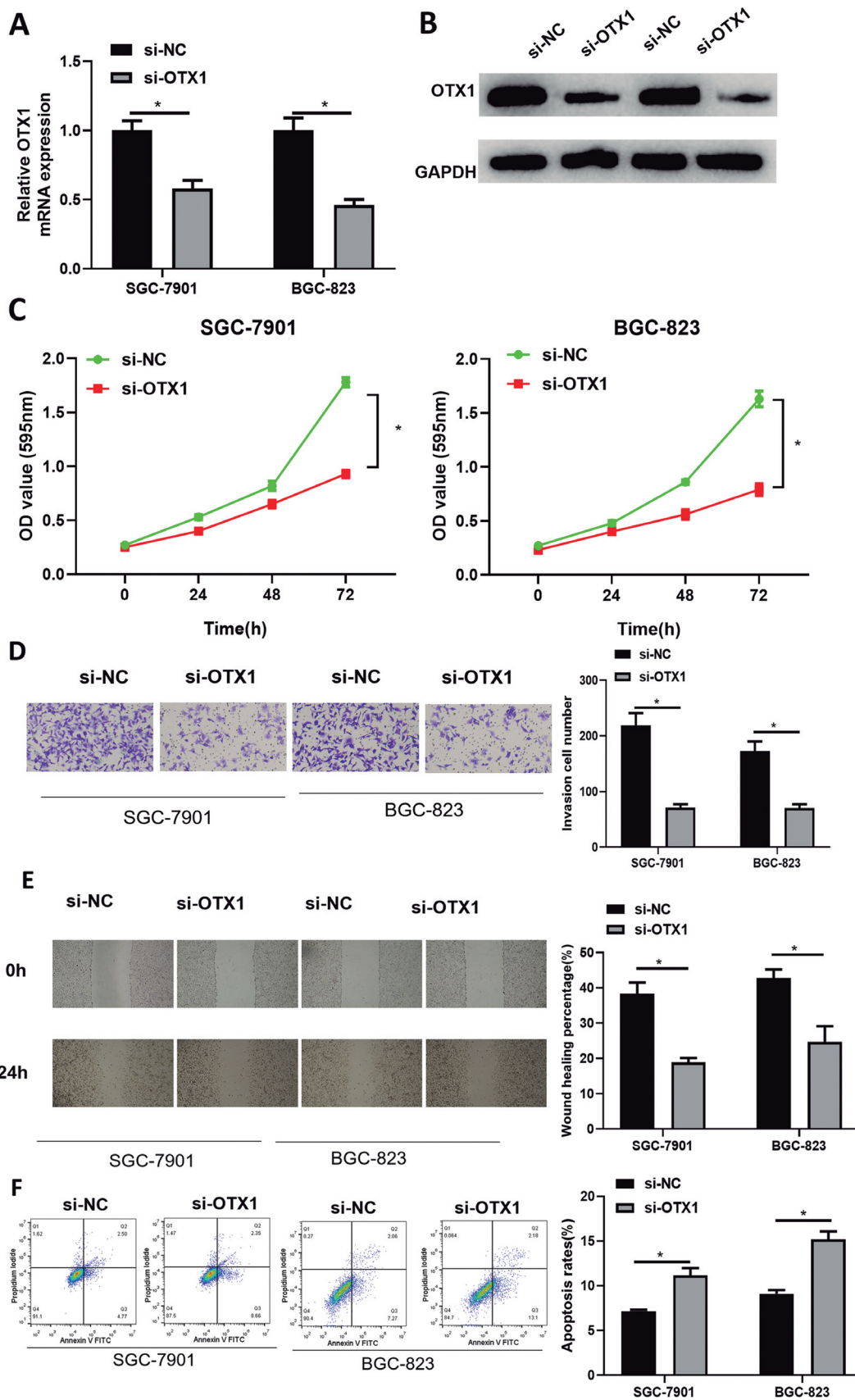
miR-195-5p noticeably restrained OTX1 mRNA and protein levels in cancer cells. Meanwhile, enforced miR-195-5p and OTX1 levels restored OTX1 expression levels (Fig. 5A,B). MTT assay result displayed that forced miR-195-5p level dramatically reduced GAC cell proliferation, while simultaneous overexpressive miR-195-5p and overexpressive OTX1 could reduce GAC cell proliferative capacity (Fig. 5C). Transwell and cell scratch assays unveiled that invasion and migration of SGC-7901 cells were inhibited after overexpressing

miR-195-5p. However, when both miR-195-5p and OTX1 were overexpressed, inhibition impact of miR-195-5p on cancer cells was weakened (Fig. 5D,E). Additionally, flow cytometry result confirmed that increased miR-195-5p level noticeably facilitated apoptosis, while increased levels of miR-195-5p and OTX1 concurrently restored cell apoptosis level (Fig. 5F). Western blot analysis of EMT-related protein expression showed that in miR-mimic+oe-NC group, E-cadherin protein level was higher than that in the miR-



**Fig. 3.** MiR-195-5p targets OTX1 in GAC cells. **A.** Differential analysis results of mRNA-seq data in TCGA-STAD are shown in the volcano map (red: up-regulated genes; green: down-regulated genes). **B.** Venn diagram of target genes of miR-195-5p predicted by TargetScan, miRWalk, miRDB, mirDIP, Starbase and other databases, and differentially up-regulated mRNAs. **C.** Correlation analysis between miR-195-5p and predicted target genes. **D.** The violinplot of OTX1 level in adjacent (yellow) and cancer (green) tissues. **E.** TargetScan website predicted targeting binding of miR-195-5p to OTX1. **F.** Dual-luciferase assay assessed luciferase activity in cells of varying groups. **G and H.** mRNA and protein levels of OTX1 in varying groups. \* $p < 0.05$ .

## MiR-195-5p/OTX1 axis restrained the development of GAC



**Fig. 4.** OTX1 knockdown inhibited GAC cell development.

**A and B.** Expression of OTX1 mRNA and protein in varying transfection groups. **C-F.** Cell proliferation, invasion (100 $\times$ ), migration (40 $\times$ ), and apoptosis in varying transfection groups, respectively. \* $p < 0.05$ .

## MiR-195-5p/OTX1 axis restrained the development of GAC

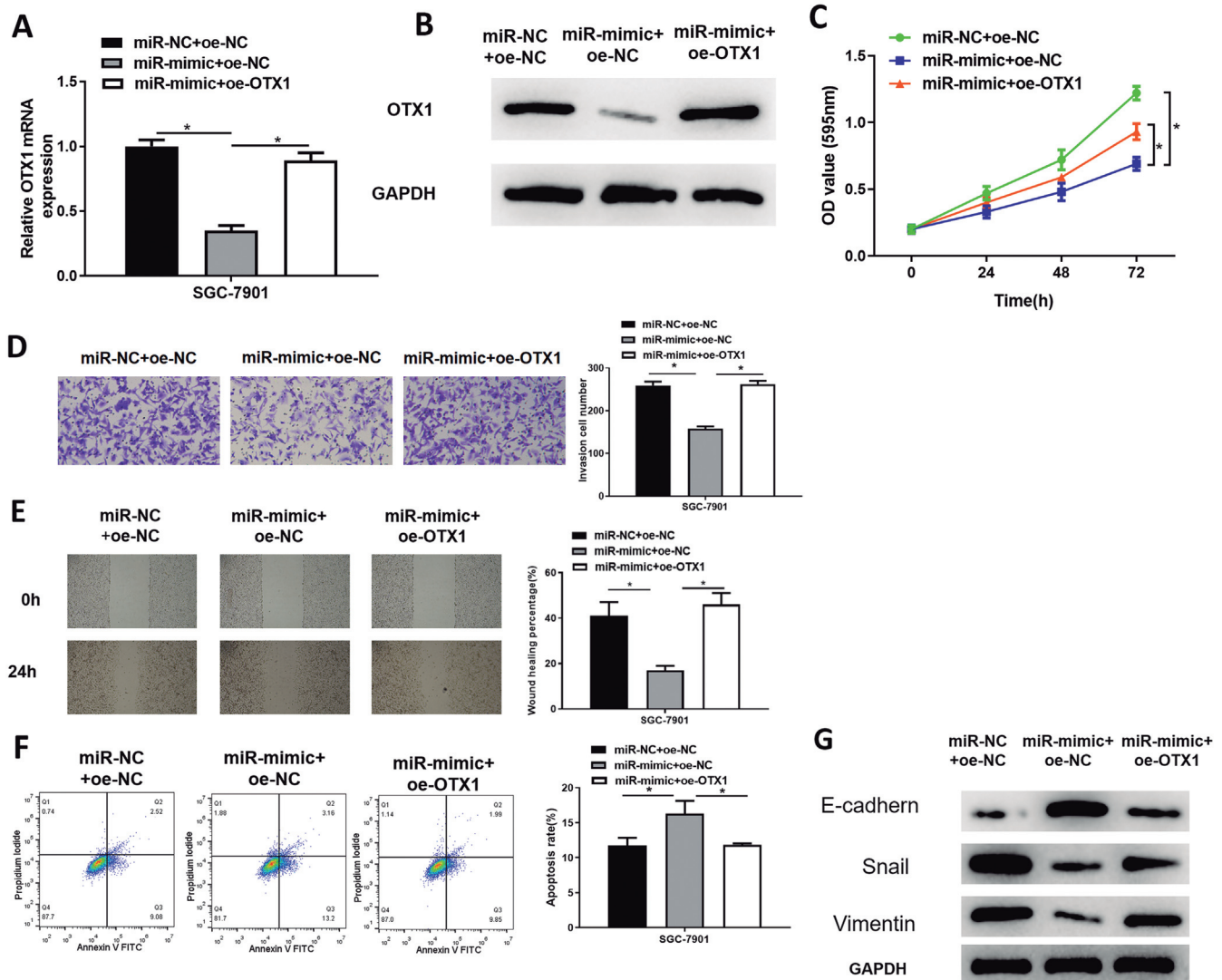
NC+oe-NC group, while Snail and Vimentin protein levels were reduced relevant to those in the miR-NC+oe-NC group. Meanwhile, in the miR-mimic+oe-OTX1 group, E-cadherin level was lower, and Snail and Vimentin levels were prominently higher than in the miR-mimic+oe-NC group (Fig. 5G). Hence, miR-195-5p partially restrained GAC cell development and promoted cell apoptosis via down-regulating OTX1 expression.

### Discussion

Recently, increasing studies have proved that miRNA is an imperative regulator affecting GAC progression (Duan et al., 2014; Kang et al., 2015). Previous studies have demonstrated that in colorectal

cancer, a significantly low miR-195-5p level enhances chemotherapy sensitivity and cancer cell apoptosis (Feng et al., 2018). Additionally, aberrancy in miR-195-5p level is observed in several other tumors, such as reduced miR-195-5p level in melanoma (Chai et al., 2018) and cervical cancer (Liu et al., 2020).

As previously studied, miR-195-5p level is remarkably reduced in GC tissue, and overexpressed miR-195-5p promotes GC cell sensitivity to chemotherapy (Nie et al., 2018). Here, we analyzed miR-195-5p level in normal and GAC cell lines and manifested that miR-195-5p was significantly under-expressed in GAC cells. Our results are congruous with those in the literature above. Besides, we investigated its biological function. Previous studies have shown that



**Fig. 5.** MiR-195-5p represses GAC cell development by targeting OTX1. **A and B.** mRNA and protein levels of OTX1 in varying transfected groups. **C-F.** Cell proliferation, invasion (100 $\times$ ), migration (40 $\times$ ), and apoptosis in varying transfection groups, respectively. **G.** E-cadherin, Vimentin and Snail levels in varying transfection groups. \* $p < 0.05$ .



## MiR-195-5p/OTX1 axis restrained the development of GAC

miR-195-5p overexpression in A549 cells can constrain proliferation, induce G0/G1 phase arrest and apoptosis (Zheng et al., 2019). Therefore, we completed functional experiments and showed that miR-195-5p was significantly under-expressed in GAC, and over-expressed miR-195-5p was able to inhibit malignant progression and EMT process of GAC cells, and promote cell apoptosis. Our results are consistent with the above research results.

To probe the downstream modulatory mechanism of miR-195-5p, public databases were applied to predict downstream targets of miR-195-5p, and negative relation of OTX1 and miR-195-5p was the highest. The dual-luciferase assay confirmed the targeting to OTX1 by miR-195-5p. OTX1 plays a role as an oncogene in many cancers. Overexpression of OTX1 facilitates cell proliferation and invasion, tumor growth in colorectal cancer, while silencing of OTX1 hampers proliferation and invasion of cancer cells (Yu et al., 2014). Besides, OTX1 is increased in hepatocellular carcinoma, with a positive correlation with lymph node metastasis and TNM stage (Li et al., 2016). The results here proved that OTX1 was significantly overexpressed in GAC, and knockdown OTX1 restrained malignant phenotypes of GAC cells. Subsequently, we found through the rescue experiment that enforced miR-195-5p level restrained cancer cell phenotype progression, while simultaneous upregulation of OTX1 and miR-195-5p rescued the above-mentioned effects via upregulating miR-195-5p. Our findings demonstrated that miR-195-5p partially constrained development of GAC cells via targeting OTX1 expression.

Overall, miR-195-5p was significantly under-expressed in GAC and OTX1 was significantly overexpressed. MiR-195-5p hampered malignant development of GAC cells through targeting OTX1. Our study suggests that miR-195-5p is a possible target for GAC, and that the miR-195-5p/OTX1 signaling axis is potentially a novel network that regulates GAC cell progression. However, there are inevitably some limitations in this study, such as lack of *in vivo* validation and clinical tissue samples to assess relevant gene levels in tissues. Therefore, *in-depth* studies are warranted to clarify exact function of miR-195-5p in proliferation, metastasis and downstream signaling pathways of GAC *in vivo*, thus providing a theoretical basis for clinical practice. In addition, miR-195-5p may have many other targets affecting the carcinogenesis of GAC. Therefore, the molecular mechanism and signaling pathway of miR-195-5p in this cancer also need further study.

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request.

*Competing interest.* The authors declare no conflicts of interest.

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*Authors' contributions.* SH and HZ contributed to the study design. XZ conducted the literature search. FQ acquired the data. CJ wrote the article. SH performed data analysis and drafted. SH revised the article. All the authors gave the final approval of the version to be submitted.

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