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E2F1-induced upregulation of lncRNA HCG18 stimulates proliferation and migration in gastric cancer by binding to miR-197-3p

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Abstract. – OBJECTIVE: LncRNA HCG18 is considered to be an oncogene in many types of tumors. The aim of this study was to explore the role of lncRNA HCG18 in gastric cancer (GC).

PATIENTS AND METHODS: HCG18 levels in GC tissues were detected. Potential biological influences of HCG18 on GC cell phenotypes were examined by Cell Counting Kit-8 (CCK-8), wound healing and transwell assay. Subsequently, bioinformatics analysis, Chromatin immunoprecipitation (ChIP), Luciferase assay and rescue experiments were conducted to identify the regulatory network of HCG18 in GC.

RESULTS: It was found that HCG18 was upregulated in GC samples, and the knockdown of HCG18 inhibited proliferative and migratory abilities in GC. The transcription factor E2F1 could directly bind to the promoter region of HCG18 and thus activate its transcription. In addition, HCG18 sponged miR-197-3p to stimulate the malignant development of GC.

CONCLUSIONS: HCG18 is upregulated in GC samples by E2F1 induction, which stimulates proliferative and migratory abilities in GC by binding to miR-197-3p.

Key Words:
GC, E2F1, HCG18, Proliferation, Migration.

Introduction

Globally, GC, originating from the gastric mucosal epithelium, has the fifth highest incidence and the third highest mortality in tumors[1]. Histologically, gastric adenocarcinoma is the most popular subtype of GC, and the prognosis in GC is relatively poor. In Europe and the United States, the long-term survival rate of GC is only 10-25% despite the great progress made on its therapeutic strategies[2]. Due to the lack of highly sensitive and specific indicators, the early diagnosis of GC is challenging. Seriously, clinical symptoms of GC appear late, which limits the surgical opportunity. Advanced GC is the major reason for the high mortality[3]. Therefore, it is urgent to develop biological markers that can effectively predict and diagnose GC.

In recent years, a novel type of non-coding RNA, lncRNA, has been discovered due to the popularity of high-throughput sequencing technology and publicity of a large number of genetic testing data. As non-coding RNAs, lncRNAs are once considered as transcription noises. They are longer than 200 nt, and mainly expressed in the cytoplasm or nuclei. Functionally, lncRNAs are capable of influencing gene expressions by epigenetic, transcriptional or post-transcriptional regulations[4]. LncRNAs have been identified to be vital regulators in tumor development. In primary non-muscle invasive bladder cancer, lncRNA UNMIBC is upregulated and stimulates cancer growth[5]. LncRNA uc.134 inhibits CUL4A-mediated LATS1 ubiquitination and triggers YAPS127 phosphorylation, thus alleviating liver cancer progression[6]. LncRNA PEG10 is upregulated in esophageal cancer tissues, and knocking down lncRNA PEG10 induces apoptosis and inhibits cancer cell growth and invasiveness[7]. Huang et al[8] have reported that HCG18 is upregulated in HCC tissues, and that it can stimulate the development of bladder cancer by binding miR-34c-5p[9], but its role in the development of GC remains largely unclear.

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GC tissues and cell lines were collected in this paper. The regulatory effects of HCG18 on malignant phenotypes of GC were first examined. Later, its potential mechanism in influencing GC development was specifically explored.

Patients and Methods

Sample Collection

A total of 28 paired GC and paracancerous tissues were collected from GC patients undergoing surgery in the First Affiliated Hospital of Jinzhou Medical University from September 2017 to September 2019. Tissue samples were pathologically confirmed and stored at -80°C. The tumor pathological classification and staging standards were all in accordance with the staging standards of the Union for international cancer control (UICC). All participants were not treated with radiotherapy and chemotherapy before operation. The gastric tumor tissues were confirmed by two pathologists in our hospital. Other kinds of tumors that migrated to the stomach were excluded. This study was approved by Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University and it was conducted after the informed consent was obtained from each subject in accordance with the Declaration of Helsinki.

Cell Culture

GC cell lines (AGS and HGC-27) provided by Cell Bank, Shanghai were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNAs extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into cDNAs using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. Primer sequences were as follows: HCG18: 5'-ATCCTGCCAATAGATGCTGCTCAC-3' (forward) and 5'-AGCCACCTTGGTCTCCAGTCTC-3' (reverse), GAPDH: 5'-AGCCACATCGCTCAGACAC-3' (forward) and 5'-GCCCAATACGACCAAATCC-3' (reverse), MiR-197-3p: 5'-GCCGGGTTGTAAACGATCCTACGG-3' (forward) and 5'-GTGATTCCGTGTCGTTAGTGG-3' (reverse), and U6: 5'-GCTTCGGCAGCACATATACTAAAAT-3' (forward) and 5'-CGCTTCAGAATTTGCGTGTCAT-3' (reverse).

Transfection

Cells were transfected with plasmids constructed by GenePharma (Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was tested by qRT-PCR.

Cell Counting Kit (CCK-8) Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (RiboBio, Guangzhou, China) for plotting the viability curves.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

Cells were pre-inoculated in a 24-well plate (2×10^4 cells/well) and incubated in 4% methanol for 30 min, followed by 10-min permeabilization in 0.5% Triton X-100, and 30-min reaction in 400 μL of 1×ApollorR. Afterwards, the cells were dyed in 1×Hoechst 33342 for another 30 min, and EdU-positive cells were calculated.

Transwell Assay

A total of 1×10^5 cells suspended in 100 μL of serum-free medium were seeded in the upper layer of a transwell chamber that was inserted in a 24-well plate, and 750 μL of medium was applied per well. On the other day, bottom cells were fixed in methanol for 15 min, stained in crystal violet in 20 min and captured using a microscope. Finally, migratory cells were counted in 5 random fields per sample.

Luciferase Assay

The JASPAR CORE database (http://jaspar.genereg.net/) was used to analyze the promoter sequences of HCG18. Sequences of E1, E2 and E3 fragments of HCG18 were synthetized and inserted into pGL3 basic vectors, respectively. After 48-h co-transfection, cells were lysed for measuring the luciferase activity, and each measurement was performed in triplicate.

Subcellular Distribution

The cells were lysed on ice in RLA for 20 min and centrifuged at 3000 r/min for 15 min. The supernatant contained the cytoplasmic fraction. Subsequently, the precipitant was washed in RLA for three times, incubated with radioimmunoprecipitation assay (RIPA) on ice for 20 min, with 30 s vortex oscillation every 5 min, and centrifuged at 12500 r/min for 15 min. Nuclear fraction was collected in the supernatant, and each fraction protein was finally determined by qRT-PCR.

Chromatin Immunoprecipitation (ChIP)

ChIP assay was conducted using EZ-Magna ChIPTM G (Millipore, Billerica, MA, USA). Cells inoculated in 10-cm culture dishes were incubated with 280 μL of 37% formaldehyde for 10 min, sonicated (10 s × 5) and centrifuged at 4°C, 12,000 r/min for 10 min. After removal of insoluble precipitation, 2 μL of antibodies and 20 μL of magnetic bead suspension were rotated with the mixture at 4°C overnight. At last, immunoprecipitation enrichment was determined by qRT-PCR with the input as a negative control.

Statistical Analysis

GraphPad 7.0 (La Jolla, CA, USA) was used for data analysis. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the two-tailed *t*-test. Pearson correlation test was applied for assessing correlation between two genes. $p<0.05$ represented that the difference was statistically significant.

Results

Upregulated HCG18 In GC Was Linked to Tumor Grade

Compared with that in paracancerous tissues, HCG18 was markedly upregulated in GC tissues (Figure 1A). Classified by pathological staging and histological subtyping, it was found that the expression of HCG18 was upregulated in advanced GC compared with that in early stage GC (Figure 1B), which was also upregulated in diffuse GC compared with that in intestinal GC (Figure 1C).

Silence of HCG18 Inhibited Proliferative and Migratory Abilities In GC

To explore the biological functions of HCG18, si-HCG18 was constructed. Transfection with si-HCG18 effectively downregulated HCG18 in both AGS and HGC-27 cells (Figure 2A). After silence of HCG18, decreased viability and EdU-positive cell ratio in GC cells suggested the inhibited proliferative ability (Figure 2B-2E). In addition, migratory cell number was decreased in GC cells transfected with si-HCG18, which indicated the suppressed migratory ability (Figure 2F). The above data demonstrated that HCG18 could promote proliferative and migratory abilities in GC.

HCG18 Level In GC Was Regulated by E2F1

A previous study showed the vital function of E2F1 in lncRNA dysfunction[10]. The JASPAR CORE database (http://jaspar.genereg.net/) was used to analyze the promoter sequence of HCG18. Three potential binding sites for E2F1 were found in the promoter region of HCG18 (Figure 3A). To further determine the specific binding sites, the Luciferase vectors containing three binding sites were constructed and inserted into the PGL3 vectors, respectively. Luciferase assay uncovered that silence of E2F1 remarkably decreased Luciferase activity in E1 region of HCG18 in HGC-27 cells (Figure 3B). In addition, ChIP assay showed that E1 region of HCG18 was abundantly enriched in anti-E2F1 than that of anti-IgG, further confirming the interaction between E2F1 and E1

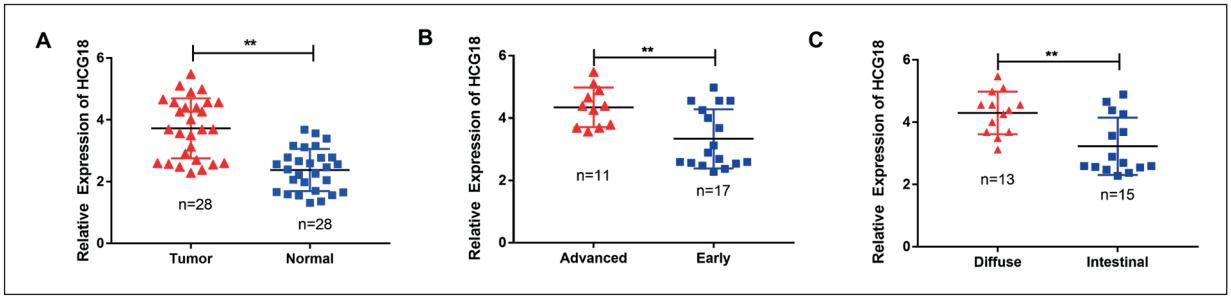

Figure 1. Upregulated HCG18 in GC was linked to tumor grade. **A,** HCG18 levels in GC tissues and paracancerous tissues. **B,** HCG18 levels in advanced GC and early-stage GC. **C,** HCG18 levels in diffuse GC and intestinal GC. $^{**}p<0.01$.

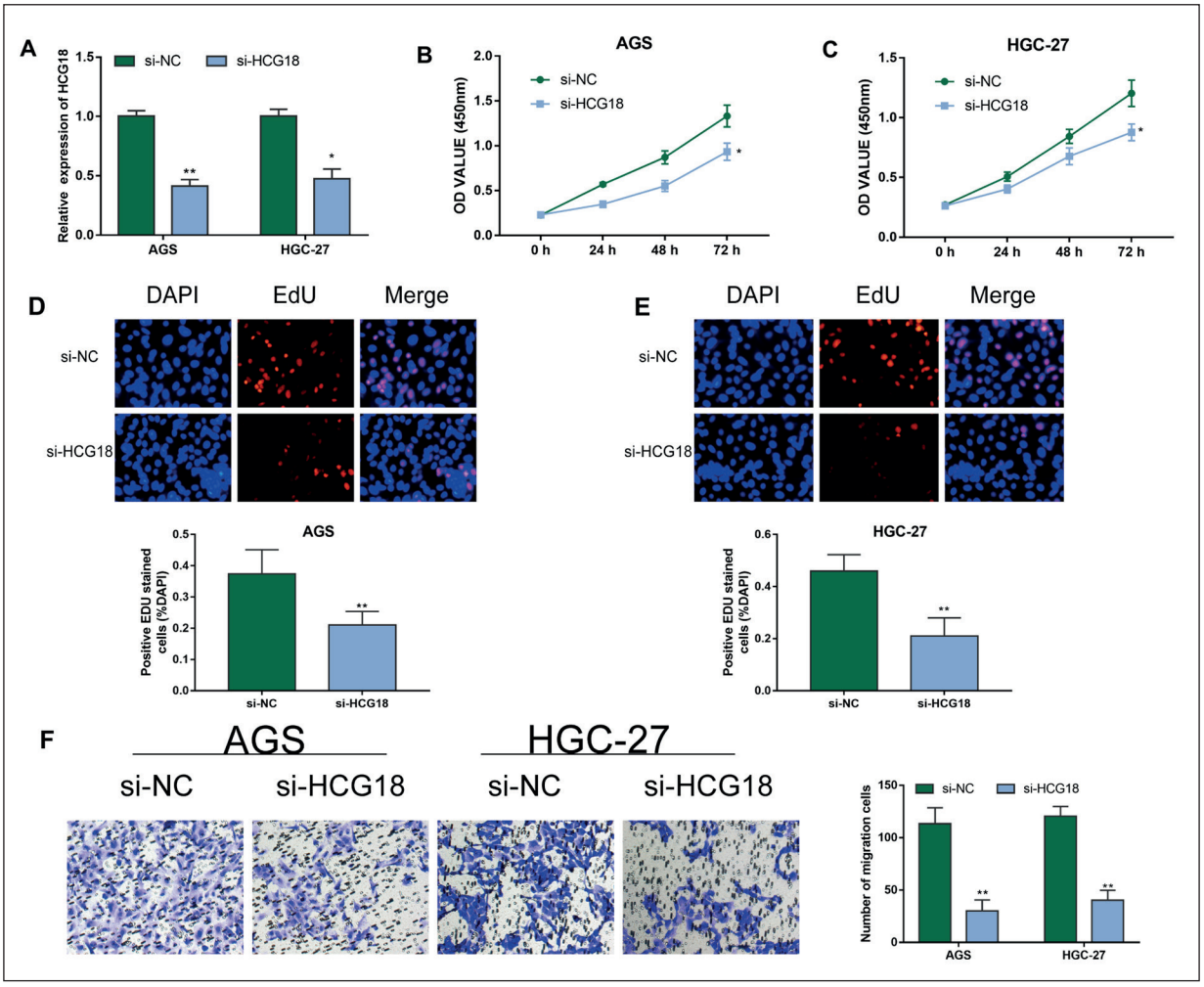

Figure 2. Silence of HCG18 inhibited proliferative and migratory abilities in GC. **A,** Transfection efficacy of si-HCG18 in AGS and HGC-27 cells. **B, C,** Viability in AGS and HGC-27 cells transfected with si-NC or si-HCG18. **D, E,** Positive EdU-stained cells in AGS and HGC-27 cells transfected with si-NC or si-HCG18 (magnification: 200×). **F,** Number of migratory cells in AGS and HGC-27 cells transfected with si-NC or si-HCG18 (magnification: 200×). **p*<0.05, ***p*<0.01.

region of HCG18 (Figure 3C). After that, two E2F1 siRNAs were constructed, and the efficacy in GC cells was tested (Figure 3D). Notably, HCG18 was markedly downregulated in GC cells transfected with either si-E2F1 #1 or si-E2F1 #2 (Figure 3E).

HCG18 Regulated Downstream Target MiR-197-3p

Studies[11,12] have shown that lncRNAs sponge miRNAs to influence expressions of miRNA downstream targets, that is, the ceRNA hypothesis. It was discovered that HCG18 was mainly distributed in the cytoplasm of GC cells (Figure 4B). The binding sequences in the 3'UTR of HCG18 and miR-197-3p were predicted online (StarBase 3.0, http://starbase.sysu.edu.cn/index.php) (Figure 4A). Their binding relationship was further confirmed by Luciferase assay (Figure 4C). In GC tissues, miR-197-3p was downregulated and negatively correlated with HCG18 level (Figure 4D, 4E).

MiR-197-3p Abolished the Regulatory Effects of HCG18 on GC Cell Phenotypes

Previous experiments showed that the proliferative and migratory abilities of AGS and HCG-27 cells were weakened after transfection of si-HCG18. Thereafter, rescue experiments were designed to clarify the role of miR-197-3p in GC. Decreased viability and positive EdU-stained cell ratio in GC cells with HCG18 were partially re-

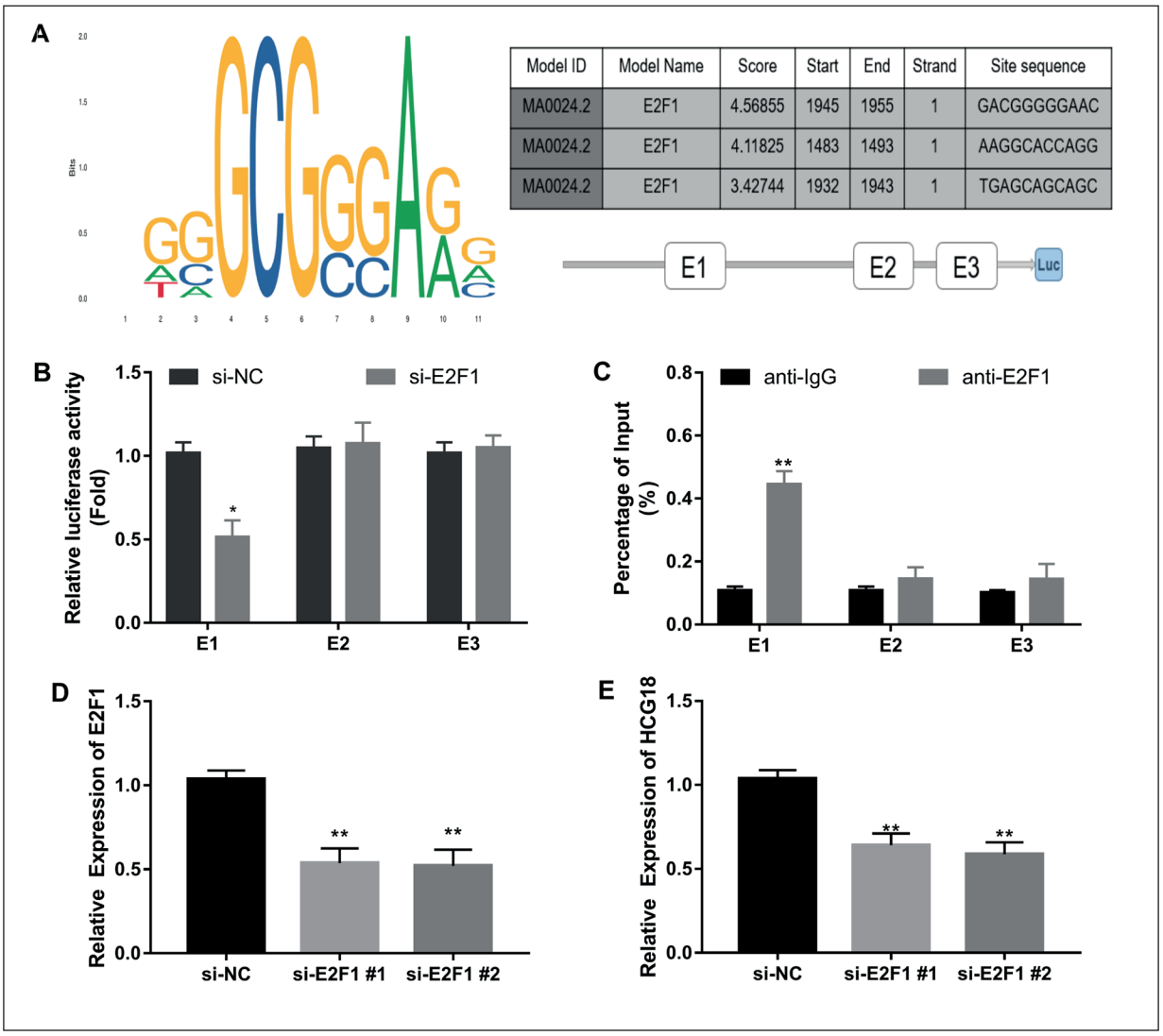

Figure 3. HCG18 level in GC was regulated by E2F1. **A,** Three potential binding sites for E2F1 in the promoter region of HCG18. **B,** Luciferase activity in three different regions of HCG18 in HGC-27 cells transfected with si-NC or si-E2F1. **C,** Immunoprecipitants of three different regions of HCG18 in anti-IgG or anti-E2F1. **D,** Transfection efficacy of si-E2F1 #1 or si-E2F1 #2 in HGC-27 cells. **E,** HCG18 level in HGC-27 cells transfected with si-NC, si-E2F1 #1 or si-E2F1 #2. *p<0.05, **p<0.01.

versed by co-silence of miR-197-3p and HCG18 (Figure 5A-D). Similarly, the inhibited migratory ability after knockdown of HCG18 in GC cells was abolished by silence of miR-197-3p (Figure 5E, 5F). Collectively, HCG18/miR-197-3p axis was responsible for stimulating proliferative and migratory abilities in GC.

Discussion

GC is the fifth prevalent malignant tumor in the world[1], whose global mortality is estimated to be 1/12. The lack of effective treatments for advanced tumors greatly attributes to tumor death. LncRNAs have been well concerned in tumor researches, and they are extensively involved in tumor development[13]. In NSCLC, upregulated lncRNA ANRIL is linked to TNM staging and prognosis[14]. LncRNA HOTAIR is highly expressed in metastatic breast cancer, and its level in primary breast cancer may be a predictive hallmark for cancer metastasis and death[15]. LncRNA HCG18 is an immune-associated gene. Wang et al[16] demonstrated that HCG18 is highly expressed in sarcomas and can be used as a

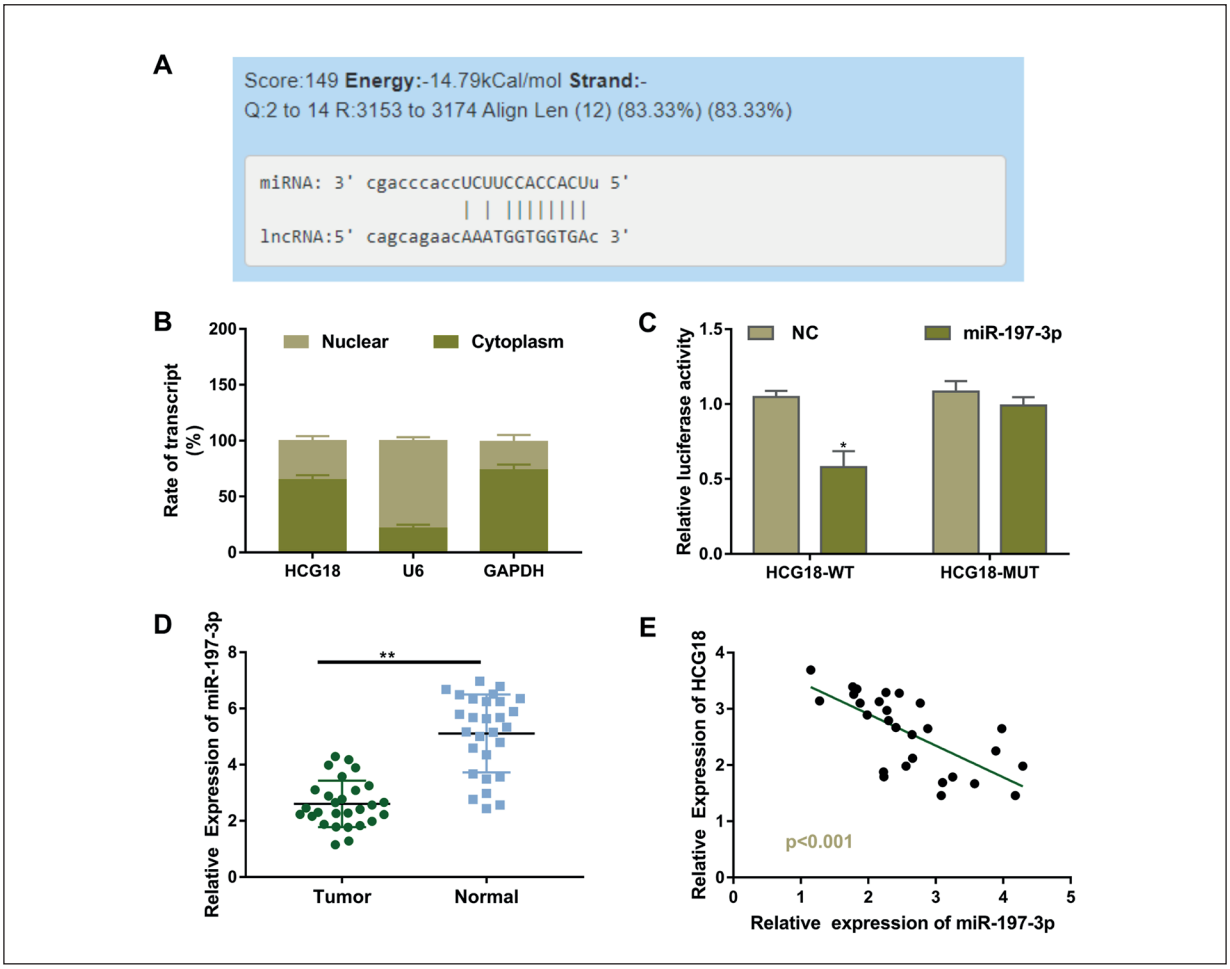

Figure 4. HCG18 regulated downstream target miR-197-3p. **A,** Binding sequences in the 3'UTR of HCG18 and miR-197-3p predicted in Starbase. **B,** Subcellular distribution of HCG18 in HGC-27 cells. **C,** Luciferase activity in HGC-27 cells co-transfected with HGC18-WT/HGC18-MUT and NC/miR-197-3p mimics. **D,** MiR-197-3p levels in GC tissues and paracancerous tissues. **E,** A negative correlation between expression levels of miR-197-3p and HCG18 in GC tissues. *$p<0.05$.

prognostic marker. HCG18/miR-146a-5p/TRAF6 is able to facilitate the intervertebral disc degeneration[17]. In bladder cancer[9], HCG18 regulates the NOTCH1 signaling by interacting with miR-34c-5p, and they synergistically regulate cancer cell phenotypes.

In this paper, HCG18 was first discovered to be highly expressed in GC samples. Subsequent investigations indicated the promotive effects of HCG18 on proliferative and migratory abilities in GC. To further identify the reason for dysregulated HCG18 in GC, bioinformatics analysis was conducted. Interestingly, the binding sites were found between E2F1 and the promoter region of HCG18. Luciferase assay and ChIP assay further demonstrated the interaction between E2F1 and HCG18. E2F1, a member of the E2F transcription factor family, is involved in many cellular processes[18], such as cell cycle, DNA synthesis and cell growth. Studies[19-21] have found that E2F1 is involved in the progression of different types of tumors. Additionally, it was identified that miR-197-3p was the downstream target of HCG18, which was negatively correlated with HCG18 in GC cells. Chen et al[22] reported that miR-197-3p exerts an anti-cancer role in the development of GC. Of note, miR-197-3p could abolish the regulatory effects of HCG18 on malignant phenotypes of GC cells, so it was believed that HCG18 stimulated the malignant development of GC by targeting miR-197-3p. Above all, it was the first time to find the carcinogenic effect of non-coding RNA HCG18 in GC. Using

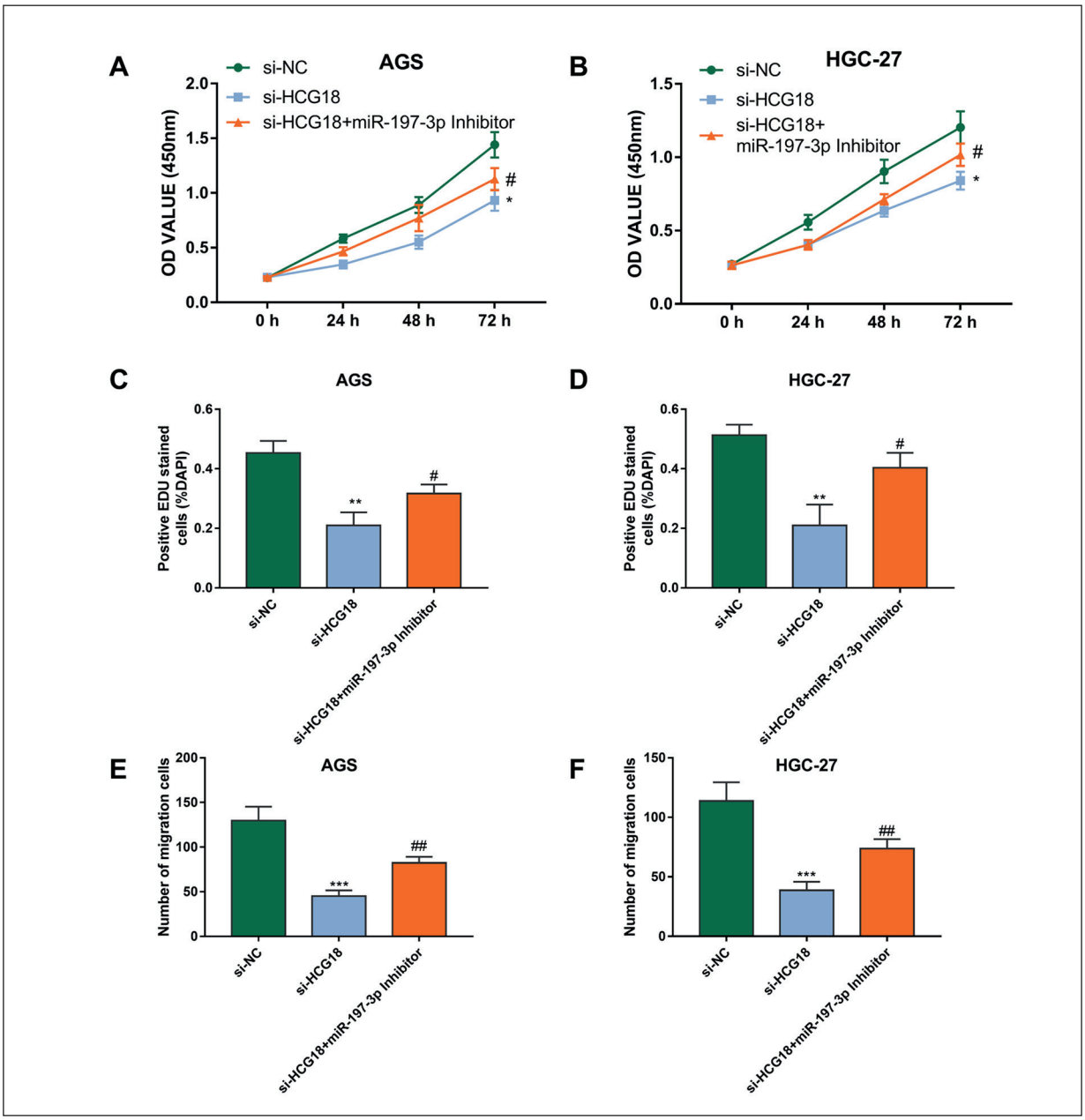

Figure 5. MiR-197-3p abolished the regulatory effects of HCG18 on GC cell phenotypes. **A, B,** Viability in AGS and HGC-27 cells transfected with si-NC, si-HCG18, or si-HCG18+miR-197-3p inhibitor. **C, D,** EdU-positive cells in AGS and HGC-27 cells transfected with si-NC, si-HCG18, or si-HCG18+miR-197-3p inhibitor. **E, F,** Number of migratory cells in AGS and HGC-27 cells transfected with si-NC, si-HCG18, or si-HCG18+miR-197-3p inhibitor. *p<0.05, **p<0.01, ***p<0.001 *vs.* si-NC group, $^{\#}p$<0.05, $^{\#\#}p$<0.01 *vs.* si-HCG18 group.

biological experimental skills, the potential mechanism of E2F1-induced upregulation of lncRNA HCG18 by binding to miR-197-3p in GC tumors was verified, which may help us to further understand the pathogenesis of GC and help diagnosis and treatment in the future.

Conclusions

HCG18 is upregulated in GC samples by E2F1 induction, which stimulates proliferative and migratory abilities in GC by binding to miR-197-3p.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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