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E2F6-mediated lncRNA CASC2 down-regulation predicts poor prognosis and promotes progression in gastric carcinoma

Yingxia Li, Libin Jiang, Shuai Lv, Haiyan Xu, Zhoupei Fan, Yixin He, Hongtao Wen*

Department of Gastroenterology, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan Province 450018, China

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ABSTRACT

Aims: To investigate the potential biological role of E2F6 and its underlying molecular mechanism in gastric carcinoma (GC) progression.

Main methods: The expressions of cancer susceptibility candidate 2 (CASC2), E2F6 and matrix metalloprotein-2 (MMP-2) were measured by quantitative real-time polymerase chain reaction and western blotting. The inhibitory effect of E2F6 on CASC2 was evaluated using luciferase reporter assay. Cell growth was assessed by colony formation assay and cell counting kit-8. Cell invasion and apoptosis were measured by transwell assay and flow cytometry assay, respectively. *In vivo* tumorigenicity was assessed by tumor xenografts in nude mice.

Key findings: Our data revealed that CASC2 was downregulated while E2F6 was upregulated in GC tissues and cell lines. Remarkably, lower expression of CASC2 was associated with poor survival in GC patients. E2F6 inhibited the expression of CASC2. Subsequently, reliable data showed that downregulation of E2F6 suppressed the proliferation and invasion, and promoted the apoptosis of GC cells. Furthermore, downregulation of E2F6 decreased the expression of MMP-2 and increased the activity of caspase-3. However, these changes triggered by E2F6 knockdown could be reversed by inhibition of CASC2. Moreover, we also proved that downregulation of CASC2 reverses the effect of E2F6 knockdown on tumor growth *in vivo*.

Significance: Our data demonstrated that E2F6 could regulate the proliferation, invasion and apoptosis of GC cells *via* inhibiting the expression of CASC2, suggesting that E2F6/CASC2 axis is another regulator of GC progression.

1. Introduction

Gastric carcinoma (GC) is one of the most common tumors in the world [1]. GC is considered as the third leading cause of cancer-related death [2]. In the past few years, though the great advance in diagnosis and therapeutic response has achieved, the prognosis of GC patients remains poor due to its delayed diagnosis [3]. Previous studies revealed that GC has been regulated by many genes, while its complex regulatory mechanisms are still not completely clear [4]. Hence, clarifying the molecular mechanism of GC is of great directive significance for searching for new biomarkers for early diagnosis, as well as for developing novel therapeutic targets.

Long non-coding RNA (lncRNAs), first described by Brannan et al. in 1990, are a class of single-stranded RNA without protein-coding capability, and longer than 200 nucleotides [5,6]. In recent years, lncRNA has been deeply studied in many diseases, including GC, and may become a useful biomarker for cancer diagnosis. As an example, Zhang et al. suggested that lncRNAs amine oxidase, copper containing 4, pseudogene, BRAF-activated noncoding RNA and LINC00857 could serve as potential diagnostic biomarkers for GC [7]. Interestingly, lncRNA has also been proved to play an important role in the progression of different cancers. Cancer susceptibility candidate 2 (CASC2), a newly described lncRNA, has been proposed to serve as a crucial regulator in human cancers [8]. CASC2 has been reported to inhibit the progression of GC *in vitro* and *in vivo via* activation of the mitogen-activated protein kinase signaling [9]. Moreover, overexpression of CASC2 could suppress angiogenesis and cell invasion in GC [10]. Such findings demonstrated the involvement of CASC2 in cancer progression, suggesting that CASC2 may act as a key player in GC. However, the regulatory mechanism of CASC2 in GC progression still needs to be more deeply explored.

E2F class of transcription factors acts as an essential regulator in cellular progression, including cell cycle and division [11]. Transcription factor E2F6, a member of the E2F family, has been proved to play a

* Corresponding author at: Department of Gastroenterology, Zheng dong hospital district of Zhengzhou University First Affiliated Hospital, 1 Middle Ring Road, Longhu District, Zhengzhou 450018, Henan Province, China.
E-mail address: wenhtgastrodep@126.com (H. Wen).

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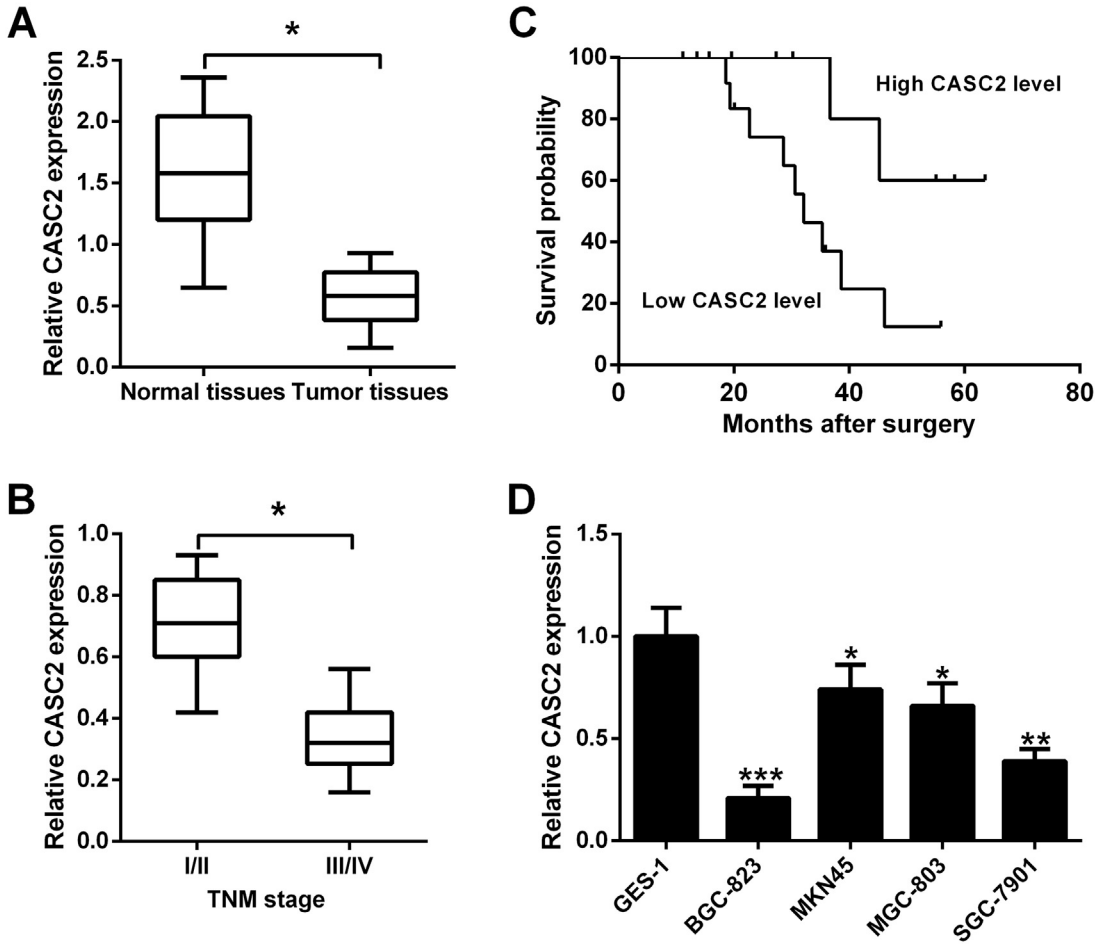

Fig. 1. The expression levels of CASC2 in GC tissues and cell lines. (A) The expression of CASC2 was detected in 37 cases of GC tissues, and matched adjacent normal tissues as a control. (B) The expression of CASC2 in GC specimens from patients with different TNM stages. (C) Survival plots of GC patients with high or low CASC2 expression analyzed using the Kaplan-Maier method. (D) The expression of CASC2 was detected in GC cell lines (BGC-823, MKN45, MGC-803, SGC-7901), and GES-1 cell as the control group. The expression of CASC2 was measured by qRT-PCR, and normalized to β-actin. All experiments were performed at least in triplicate, and data were presented as the mean ± SD, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

key role in the regulation of cancer progression. For instance, E2F6 has been documented to inhibit cell apoptosis induced by cobalt chloride-mimetic hypoxia in HEK293 cells by regulating the expression of E2F1 [12]. Moreover, Trimarchi et al. demonstrated that E2F6 was a component of the Bmi1-containing polycomb complex, and exerted its biological function *via* recruiting the polycomb transcriptional repressor complex [13]. Transcriptome and proteome analysis of GC tissues and cell lines showed that E2F6 was highly expressed in GC [14]. While the potential biological role of E2F6 in GC and its underlying mechanism remain poorly understood. In this present study, our data suggested that E2F6 could regulate the progression of GC *in vivo* and *in vitro via* regulating the expression of CASC2. Our study established a new regulatory axis in occurrence and development of GC.

2. Materials and methods

2.1. Human tissue samples

A total of 37 paired GC samples and adjacent normal tissues were collected from the First Affiliated Hospital of Zhengzhou University. The GC diagnosis was confirmed by an experienced pathologist, and all patients had not accepted any anti-cancer treatment before surgery. These samples were stored in liquid nitrogen. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University, and written informed consents were obtained from each patient before the study initiation.

2.2. Cell culture and transfection

Human gastric carcinoma cell lines (BGC-823, MKN45, MGC-803, SGC-7901) and normal gastric mucosa cell line GES-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO_2. All culture media and reagents used in this study were purchased from HyClone (Logan, UT, USA).

Empty pcDNA-3.1 (pcDNA), sh-E2F6, sh-CASC2 and sh-negative control (sh-NC) were obtained from GeneCopoecia (Guangzhou, China). The full sequence of E2F6 was sub-cloned into the pcDNA-3.1 vector (Invitrogen, Carlsbad, CA, USA). Lipofectamine 2000 (Invitrogen) was used for cell transfection according to the manufacturer's instructions.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

At 48 h after transfection, Trizol reagent (Invitrogen) was used to isolate total RNA from GC tissues or cell lines, according to the manufacturer's instructions. The expression of CASC2 and E2F6 were tested by qRT-PCR using the SYBR green method (Takara, Dalian, China) under the guidance of manual. β-actin was employed as a loading

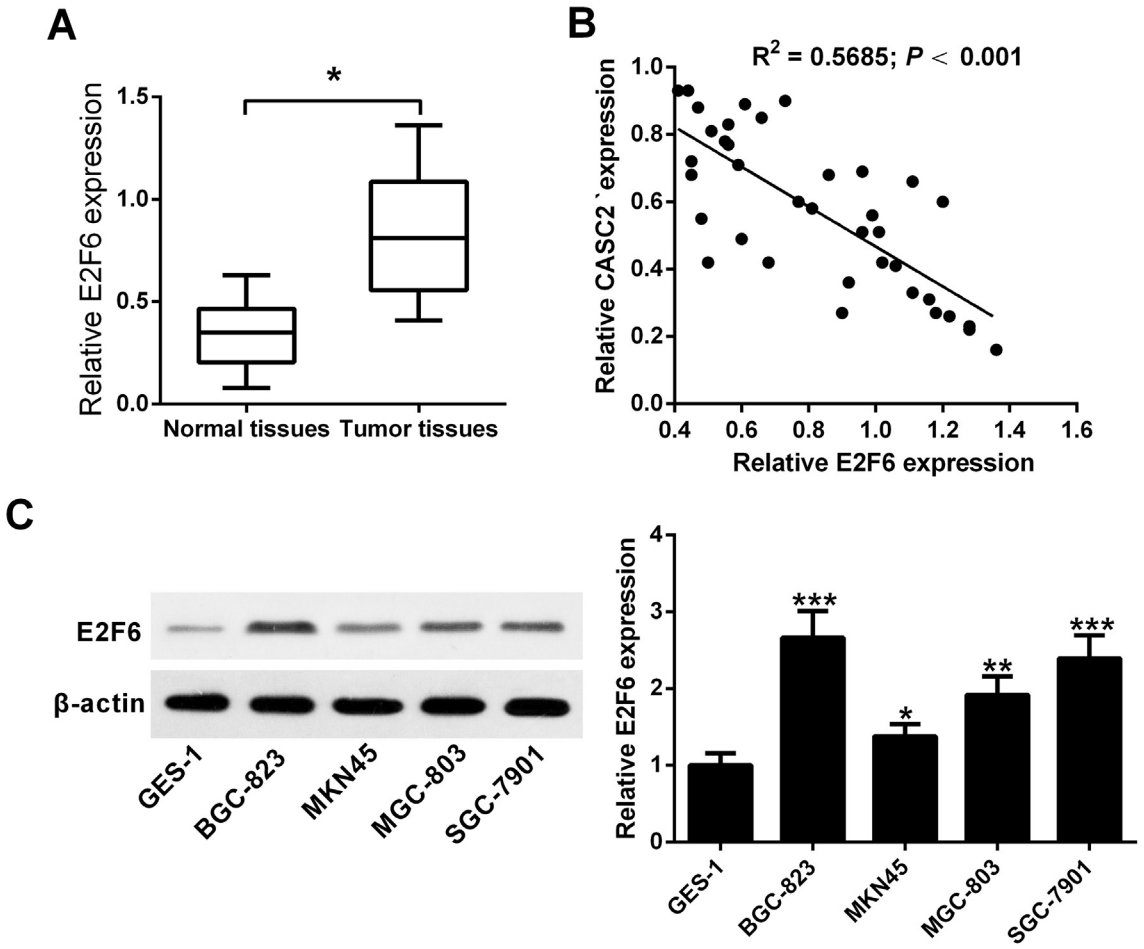

Fig. 2. The expression levels of E2F6 in GC tissues and cell lines. (A) qRT-PCR was performed to detect the expression of E2F6 in 37 pairs of GC tissues and adjacent normal tissues. (B) Correlation analysis ofCASC2 and E2F6 expression in GC tissues. (C) The expression levels of E2F6 protein were detected by WB. β-actin was served as a loading control, and all experiments were performed at least in triplicate. All data were presented as the mean ± SD, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

control. All experiments were repeated in triplicate. The relative expression of CASC2 and E2F6 were calculated and normalized by the $2^{-\Delta\Delta Ct}$ method. The primers used in this study were listed as follows: CASC2 Forward: 5′-GCACATTGGACGGTGTTTCC-3′, Reverse: 5′-CCCA GTCCTTCACAGGTCAC-3′; E2F6 Forward: 5′-GACCTCGTTTTGATGTA TCGCTG-3′, Reverse: 5′-ATACACTCTCCGCTTTCGGAC-3′; β-actin Forward: 5′-AGCCATGTACGTAGCCATCC-3′, Reverse: 5′-TCCCTCTCA GCTGTGGTGGTGAA-3′.

2.4. Western blotting (WB)

At48 h after transfection, GC cells were lysed using RIPA Lysis Buffer (Thermo Fisher, China) according to the manufacturer's introductions. Equal amount of protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). Afterward, the membrane was blocked with 5% skim milk, probed with primary antibodies overnight at 4 °C and then incubated with secondary antibody for 1 h at room temperature. The primary antibodies against E2F6, matrix metalloprotein-2 (MMP-2) or β-actin (as an internal control), and secondary antibody were obtained from Abcam (Cambridge, MA, USA), and used following the manufacturer's instructions. The bands were visualized using the ECL western blotting kit from Abcam according to the manufacturer's instructions. The concentrations of all antibodies were selected also according to the manufacturer's instructions.

2.5. Determination of caspase-3 activity

The activity of caspase-3 was determined using Caspase-3 activity assay kit (Beyotime, Shanghai, China) per manufacturer's instructions. Briefly, BGC-823 and SGC-7901 cells were collected after transfection. The cells were lysed in ice-cold lysis buffer and the extracted proteins were qualified using BCA protein assay kit (Pierce, Rockford, IL, USA). Following this, samples were incubated with acetyl-Asp-Glu-Val-Asp p-nitroanilide for 1 h and then tested for caspase-3 activity by determining the absorbance at 405 nm.

2.6. Luciferase reporter assay

The promoter region of CASC2 was fused into a luciferase reporter vector. We co-transfected wild type CASC2 reporter gene plasmid with pcDNA-E2F6 or sh-E2F6 into GC cells (BGC-823 and SGC-7901) using Lipofectamine 2000. pcDNA was the negative control for pcDNA-E2F6, and sh-NC was the negative control for sh-E2F6. At 48 h after transfection, the luciferase activity was tested by using a Dual-luciferase reporter assay system (Promega, Madison, WI, USA) under the guidance of manual.

2.7. Cell counting Kit-8 (CCK-8) assay

GC cells were transfected with sh-NC, sh-E2F6, or sh-CASC2. Cell proliferation was measured using CCK-8 (Solarbio, Beijing, China)

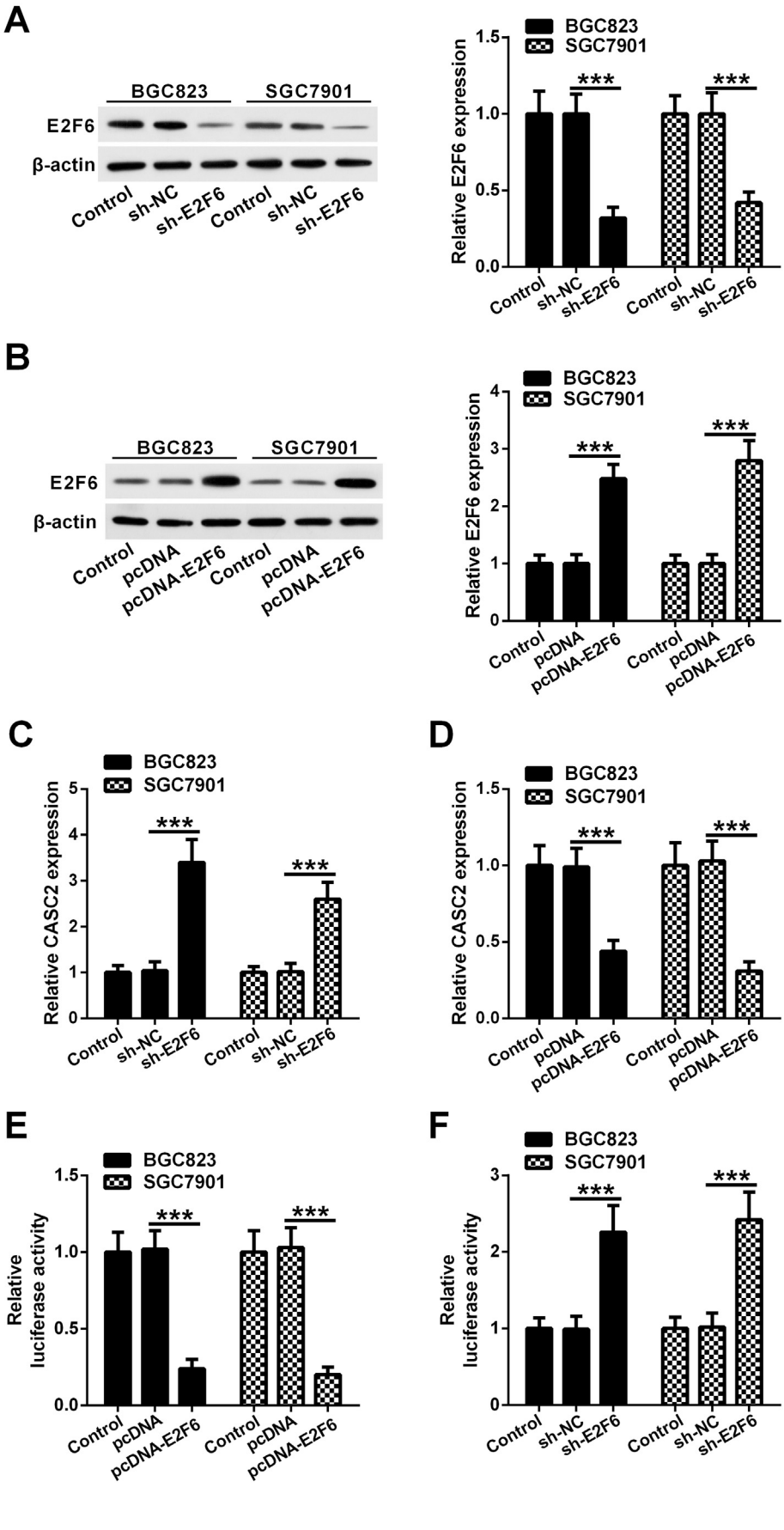

Fig. 3. CASC2 is regulated by E2F6 in GC cells. (A) The reduced expression of E2F6 in GC cells transfected with sh-E2F6. (B) The increased expression of E2F6 in GC cells transfected with pcDNA-E2F6. (C) The expression of CASC2 in GC cells transfected with sh-E2F6 was determined by qRT-PCR. (D) The expression of CASC2 in GC cells transfected with pcDNA-E2F6 was determined by qRT-PCR. (E and F) Luciferase activity of GC cells was detected following transfection of pcDNA-E2F6 or sh-E2F6. sh-NC and pcDNA-3.1 plasmids were employed as negative controls for sh-E2F6 and pcDNA-E2F6. All experiments were performed at least in triplicate. All data were presented as the mean ± SD, $^{***}p < 0.001$.

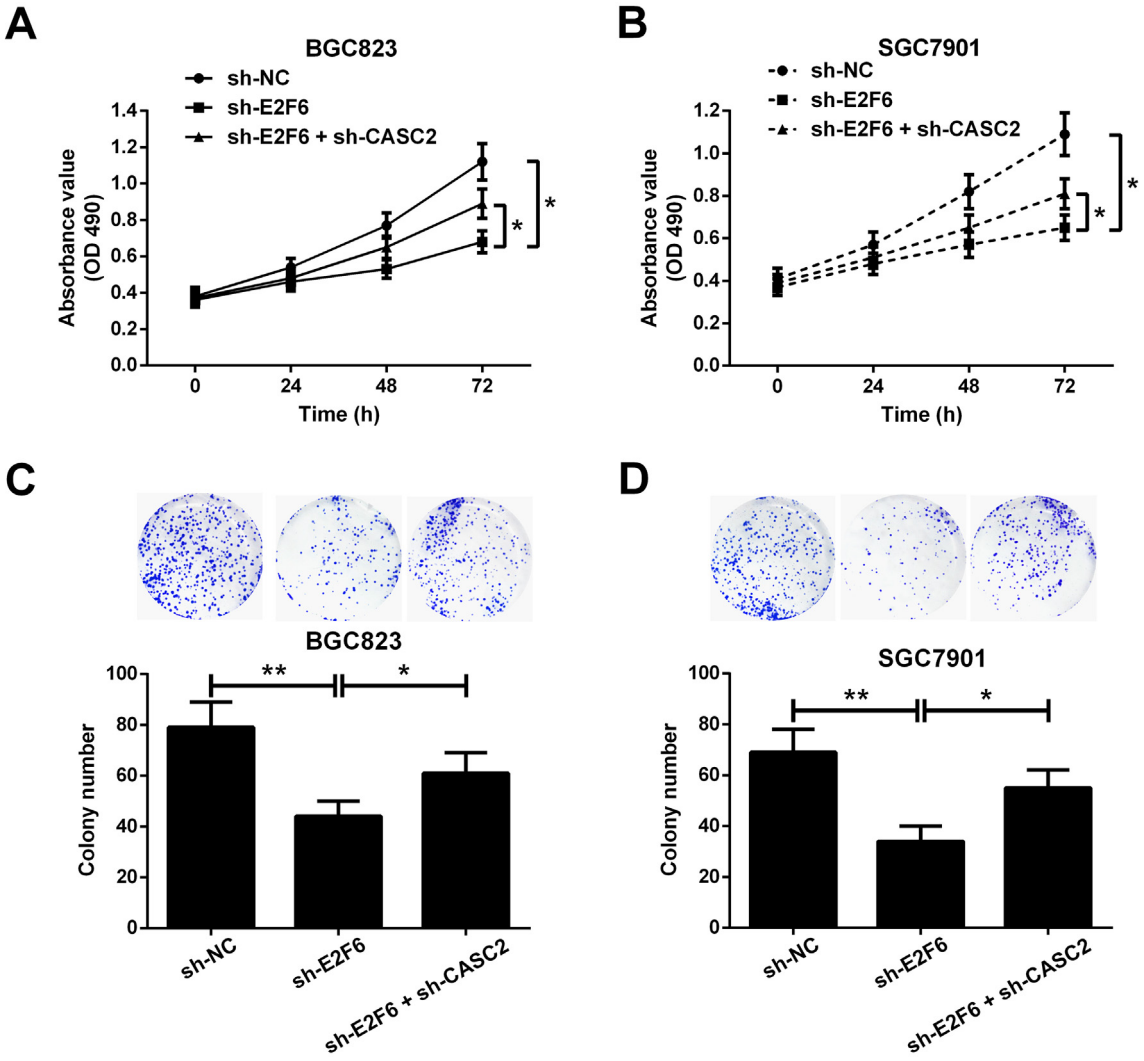

Fig. 4. Downregulation of CASC2 abrogates the effect of E2F6 knockdown on the proliferation of GC cells. BGC-823 and SGC-7901 cells were transfected with sh-NC, sh-E2F6 alone or with sh-CASC2. (A and B) CCK-8 assay was performed to detect the proliferation of BGC-823 and SGC-7901 cells. (C) The colony number of BGC-823 cells was measured. (D) The colony number of SGC-7901 cells were measured. All experiments were performed at least in triplicate. All data were presented as the mean ± SD, $*p < 0.05$ and $**p < 0.01$.

under the guidance of manual. The principle of CCK-8 assay is that dehydrogenase activities in cells could reduce water-soluble tetrazolium salt, which presents a yellow color product (formazan dye). Formazan dye is soluble in the culture medium. The amount of formazan dye is proportional to the number of living cells. Briefly, BGC-823 and SGC-7901cells (1×10^3cells/well) were seeded into a 96-well plate after transfection, and cultured for indicated times (24 h, 48 h or 72 h) under 5% CO_2/95% air at 37 °C. Subsequently, CCK-8 solution was added into each well and incubated for 2 h under 5% CO_2/95% air at 37 °C. The absorbance of each well was measured at 490 nm using a microplate reader.

2.8. Colony formation assay

At 48 h post transfection, BGC-823 and SGC-7901 cells (1×10^3) were plated on a 35-mm dish, and maintained in RPMI-1640 containing 10% FBS for 14 days at 37 °C. Afterward, the cells were washed three times with phosphate buffered saline (PBS; 0.01 M). Subsequently, visible colonies were fixed with 4% paraformaldehyde (Sigma-Aldrich, Louis, MO, USA) for 15 min, followed by staining with 0.2% crystal violet (Sigma-Aldrich) for 20 min. Finally, the colonies were photographed and colonies consisting > 50 cells were counted using Image J software (NIH, Bethesda, MD).

2.9. Transwell assay

The 24-well transwell chamber (BD Biosciences, San Jose, CA, USA) with 8-μm pore was pre-coated with Matrigel, and used to determine the invasion of BGC-823 and SGC-7901 cells. BGC-823 and SGC-7901 cells in the serum-free medium were placed in the upper chamber after transfection, and RPMI-1640 medium supplemented with 10% FBS was added into the lower chamber. After 24 h of incubation at 37 °C, the cells on the upper surface of the chamber were removed. The cells on the lower surface of the chamber were fixed with 4% paraformaldehyde, and stained with 0.2% crystal violet. Invading cells were visualized under an Olympus fluorescence microscope (Japan) and counted from 5 randomly selected fields.

2.10. Flow cytometry assay

Cell apoptosis was detected by an Annexin V-FITC apoptosis kit (Sigma-Aldrich) under the guidance of manual. BGC-823 and SGC-7901 cells transfected with sh-NC, sh-E2F6 or sh-CASC2 were collected and washed with PBS. Then the cells were re-suspended in a binding buffer,

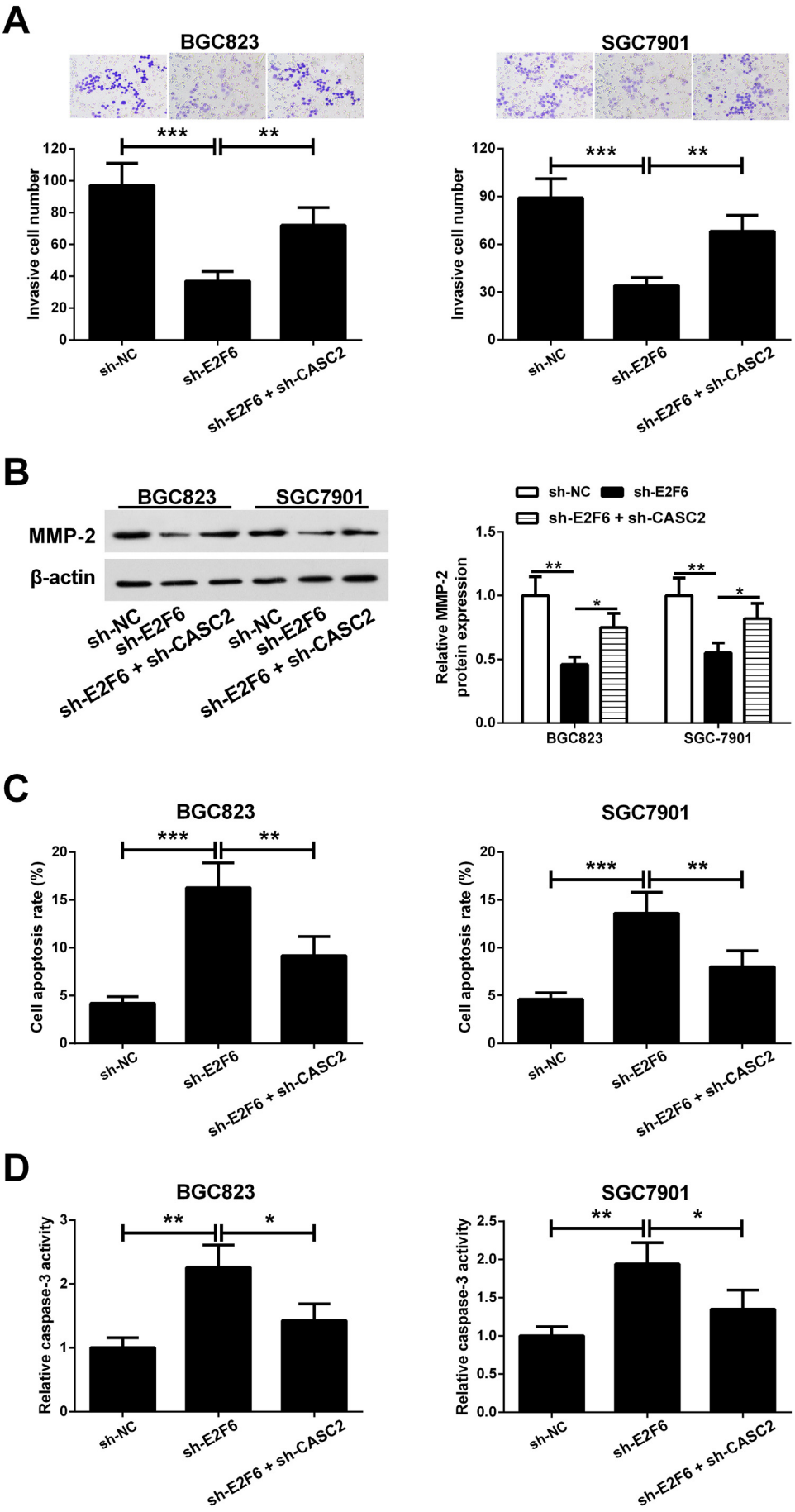

Fig. 5. Downregulation of CASC2 abrogates the effect of E2F6 knockdown on the invasion and promoted the apoptosis of GC cells. BGC-823 and SGC-7901 cells were transfected with sh-NC, sh-E2F6 alone or with sh-CASC2. (A) Transwell invasion assay was used to detect the invasion of BGC-823 and SGC-7901 cells. (B) The expression of MMP-2 in GC cells was determined by WB. (C) After transfection, the apoptosis of BGC-823 and SGC-7901 cells was measured by flow cytometry using an Annexin V-FITC apoptosis kit. (D) The caspase-3 activity in BGC-823 and SGC-7901 cells was determined using Caspase-3 activity assay kit. All experiments were performed at least in triplicate. All data were presented as the mean ± SD, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

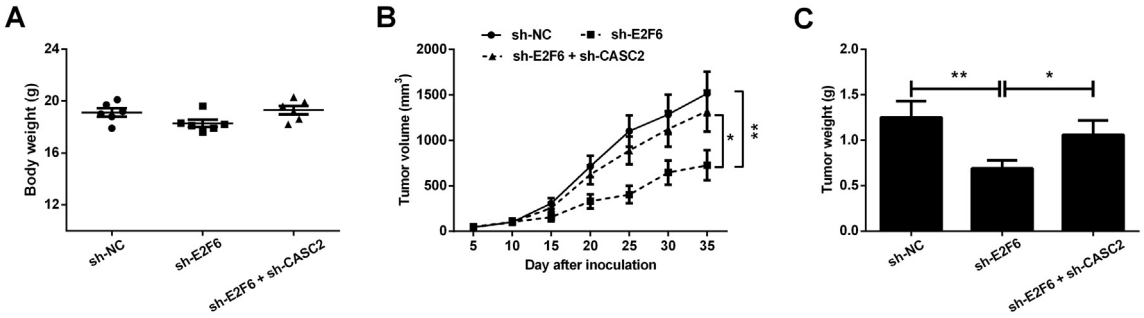

Fig. 6. Downregulation of CASC2 abrogates the effect of E2F6 knockdown on tumor growth *in vivo*. BGC-823 cells transfected with sh-NC, sh-E2F6 or sh-CASC2 were subcutaneously injected into nude mice. (A) At 35th days after injection, the body weight of mice was measured. (B) Tumor size was monitored every five days after injection. (C) The tumor weight was measured at 35th days after injection. All experiments were performed at least in triplicate. All data were presented as the mean ± SD, $*p < 0.05$, $**p < 0.01$.

followed by double staining with FITC-Annexin V and propidium iodide in the dark. Subsequently, the apoptosis of BGC-823 and SGC-7901 cells was detected by flow cytometry (BD Biosciences) using the CellQuest Pro software.

2.11. Tumor xenograft

Six-week-old male BALB/c nude mice were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). BGC-823 cells transfected with sh-NC, sh-E2F6 or sh-CASC2 were subcutaneously injected into the back of nude mice, respectively. The tumor size was detected every five days, and the tumor volume was calculated according to the formula: volume = length × width^2 × 0.5. At 35th days after injection, the body weight of mice was measured. Then the tumor tissues were taken out after execution, and the tumor weights were also tested. Our experiments were approved by the animal ethics committee of the First Affiliated Hospital of Zhengzhou University.

2.12. Statistical analysis

All statistical analyses were completed using the SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) and graphed using GraphPad Software (La Jolla, CA, USA). The data were compared using Student's *t*-test or one-way ANOVA. All data were presented as means ± standard deviation (SD), and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. CASC2 was significantly downregulated in GC tissues and cell lines

A recent study reported that the expression of CASC2 was downregulated in GC tissues and cell lines [15]. Herein, we confirmed the differential expression of CASC2 in GC by detecting the expression of CASC2 in 37 cases of GC tissues and cell lines. qRT-PCR analysis showed that CASC2 was significantly downregulated in GC tumor tissues compared with that in their adjacent normal tissues (Fig. 1A). Besides, we also investigated the expression of CASC2 in GC specimens from patients with different tumor node metastasis (TNM) stages. The result demonstrated that lower expression of CASC2 was found in GC specimens from patients with III/IV stage compared with that in patients with I/II stage (Fig. 1B). Moreover, Kaplan-Meier curve analysis showed that GC patients with high CASC2 expression exerted a high survival rate (Fig. 1C). Likewise, reduced expression of CASC2 was observed in GC cell lines (BGC-823, MKN45, MGC-803, SGC-7901) relative to normal gastric mucosa cell line GES-1 (Fig. 1D). These results proved that abnormal expression of CASC2 was closely linked with GC progression.

3.2. E2F6 was significantly upregulated in GC tissues and cell lines

We further measured the expression of E2F6 in GC tissues and cell lines. As detected by qRT-PCR, increased expression of E2F6 was found in GC tumor tissues compared with that in their adjacent normal tissues (Fig. 2A). Also, we found that there is a negative correlation between CASC2 and E2F6 expression in GC tumor tissues (Fig. 2B). Furthermore, increased expression of E2F6 in GC cell lines (BGC-823, MKN45, MGC-803, SGC-7901) was also identified by WB (Fig. 2C).

3.3. E2F6 directly regulates the expression of CASC2

To uncover the effects of E2F6 on CASC2 expression, sh-E2F6 and pcDNA-E2F6 plasmids were transfected into BGC-823 and SGC-7901 cells to alter the levels of E2F6. The results of WB demonstrated that the expression of E2F6 cells was significantly reduced in BGC-823 and SGC-7901 transfected with sh-E2F6 (Fig. 3A), but was significantly increased in BGC-823 and SGC-7901 cells transfected with pcDNA-E2F6 (Fig. 3B). Of note, downregulation of E2F6 promoted the expression of CASC2 in BGC-823 and SGC-7901 cells (Fig. 3C), and upregulation of E2F6 inhibited the expression of CASC2 in BGC-823 and SGC-7901 cells (Fig. 3D). Then we cloned the CASC2 promoter gene sequence into a luciferase reporter vector, and co-transfected wild type CASC2 reporter gene with pcDNA-E2F6 or sh-E2F6 into GC cells. Relative luciferase activity assay proved that upregulation of E2F6 decreased, but downregulation of E2F6 increased luciferase activity of CASC2 promoter luciferase reporter gene vector (Fig. 3E and F).

3.4. Downregulation of CASC2 reverses the effect of E2F6 knockdown on cell proliferation in GC cells

To identify the potential role of E2F6 and CASC2 in GC progression, we investigated the effect of E2F6 and CASC2 on GC cell proliferation. BGC-823 and SGC-7901 cells were transfected with sh-NC, sh-E2F6 alone or with sh-CASC2, and then subjected to CCK-8 assay and colony formation assay. The results of CCK-8 assay revealed that knockdown of E2F6 inhibited the proliferation of BGC-823 and SGC-7901 cells, and this action was mitigated by knockdown of CASC2 (Fig. 4A and B). Furthermore, the colony number of BGC-823 cells was reduced following E2F6 downregulation, and this effect was blocked in BGC-823 cells transfected with sh-E2F6 and sh-CASC2 (Fig. 4C). Besides, similar results were observed in SGC7901 cells (Fig. 4D). These results indicated that downregulation of CASC2 reversed the effect of E2F6 knockdown on the proliferation of GC cells.

3.5. Downregulation of CASC2 reverses the effect of E2F6 knockdown on cell invasion and apoptosis in GC cells

Subsequently, the effect of E2F6 and CASC2 on cell invasion and

apoptosis also were investigated. BGC-823 and SGC-7901 cells were transfected with sh-NC, sh-E2F6 alone or with sh-CASC2, and transwell invasion assay was carried out to measure the invasion of GC cells at 48 h after transfection. The results demonstrated that downregulation of E2F6 inhibited the invasion of BGC-823 and SGC-7901 cells, which was obviously abrogated by knockdown of CASC2 (Fig. 5A). Moreover, we found that MMP-2 was downregulated following sh-E2F6 transfection, and this action induced by sh-E2F6 was mitigated by inhibition of CASC2 (Fig. 5B). In parallel, flow cytometry analysis suggested that downregulation of E2F6 promoted the apoptosis of BGC-823 and SGC-7901 cells, and this effect was mitigated by knockdown of CASC2 (Fig. 5C). Furthermore, downregulation of E2F6 decreased the activity of caspase-3 in BGC-823 and SGC-7901 cells, which was blocked by downregulation of CASC2 (Fig. 5D). These data demonstrated that downregulation of CASC2 reversed the effect of E2F6 knockdown on cell invasion and apoptosis in GC cells.

3.6. Downregulation of CASC2 reverses the effect of E2F6 knockdown on tumor growth in vivo

Given the functional role of CASC2 *in vitro*, we further determined if CASC2 knockdown reverses the effect of E2F6 knockdown on tumor growth *in vivo*. BGC-823 cells transfected with sh-NC, sh-E2F6 alone or with sh-CASC2 were subcutaneously injected into nude mice. At 35th days after injection, the body weight and tumor weight of animals were measured, and the data showed that downregulation of either E2F6 or CASC2 had no effect on the body weight of mice (Fig. 6A). Compared with the sh-NC group, the tumor weight was markedly reduced in the sh-E2F6 group. Notably, the weight of tumors from sh-E2F6-transfected-BGC-823 cells was lighter than that from sh-E2F6 + sh-CASC2-transfected BGC-823 cells (Fig. 6B). Furthermore, the tumor size was measured every five days after inoculation. As shown in Fig. 6C, downregulation of E2F6 significantly inhibited the tumor growth, which was reversed by downregulation of CASC2 (Fig. 6C).

4. Discussion

To date, the traditional treatment method of GC includes surgery, radiotherapy, and chemotherapy [16]. Notably, the mortality rate of GC patients diagnosed at a late-stage was higher than that of patients diagnosed at an early-stage, and high recurrence rate of GC needs to be faced [17,18]. The key to improve the prognosis for GC generally lies in early diagnosis and aggressive treatment. Hence, it is an urgent need to find novel diagnostic and therapeutic approaches for GC.

LncRNAs are a class of non-coding functional RNAs, and serve as key players in numerous cellular functions. The role of lncRNA in human diseases has been widely reported. For example, lncRNA growth arrest-specific transcript 5 has been reported to enhance the G1 cell cycle arrest via Y-box binding protein 1/p21 axis in stomach cancer [19]. In addition, *in vitro* experiments suggested that upregulation of Hox transcript antisense intergenic RNA was correlated with larger tumor size, advanced stage, and metastasis, as well as poor prognosis of GC patients. Moreover, Hox transcript antisense intergenic RNA functioned as a decoy of miR-331-3p to increase the expression of human epithelial growth factor receptor 2, thereby promoting the proliferation, migration and invasion of GC cells [20]. LncRNA CASC2, a recently discovered lncRNA, has been shown to be involved in many cancers. In thyroid carcinoma, downregulation of CASC2 was predictive of multifocality, advanced TNM stage, and poor prognosis, and overexpression of CASC2 inhibited tumorigenesis, suggesting the potential role of CASC2 in tumorigenesis [21]. CASC2 was found to be downregulated in hepatocellular carcinoma. CASC2 inhibited the viability and induced the apoptosis of hepatocellular carcinoma cells *via* regulating the expression of miR-24-3p [22]. A similar role for CASC2 has been documented in colorectal cancer. Specifically, CASC2 could decrease the expression of protein inhibitor of activated STAT3 by targeting miR-18a, leading to upregulation of downstream genes of the signal transducer and activator of transcription-3, consequently suppressing tumor growth *in vitro* and *in vivo* [23]. Besides, our previous study reported that downregulation of CASC2 promoted the resistance of GC cells towards cisplatin by targeting miR-19a, indicating that GASC2 may serve as a potential target for GC treatment [24]. However, the regulation mechanism of CASC2 in GC progression is still not well known, and needs to be explored more deeply. In this present study, our data demonstrated that CASC2 was downregulated in GC tissues and cell lines. Low expression of CASC2 was correlated with advanced TNM stage and worse survival in GC, indicating that CASC2 may be a potential biomarker for GC. Upregulation of CASC2 inhibited the proliferation and invasion and promoted the apoptosis of GC cells. A recent study by another group also showed that CASC2 was downregulated in human GC tissues and cell lines and overexpression of CASC2 inhibited the proliferation of GC cells [9]. These studies suggest that downregulation of CASC2 is involved in the initiation and progression of GC.

The significance of E2F6 in cancers has been discussed in several studies, and it has been recognized as a potential biomarker for human cancers [25]. In triple-negative breast cancer, E2F6 was identified as a target of miR-185 and participated in miR-185-mediated inhibition of tumor growth *in vitro* and *in vivo* [26]. E2F6 was identified as a tumor suppressive transcription factor in human cancers by regulating its target promoters in a histone methyltransferase-independent manner [27]. Notably, E2F6 has been reported to control the expression of lncRNA H19, thereby participating in the regulation of breast cancer progression [28]. Additionally, a recent study in non-small cell lung cancer demonstrated that transcription factor E2F6 could inhibit the transcriptional expression of lncRNA LINC01436, which promoted tumor growth and metastasis *in vitro* and *in vivo* through miR-30a-3p/endothelial PAS domain-containing protein 1 axis [29]. However, the regulatory effect of E2F6 on lncRNA in GC has not been studied yet. In the present study, upregulation of E2F6 and downregulation of CASC2 were identified in GC specimens and cell lines. Moreover, our data showed that there is a negative correlation between E2F6 and CASC2 expression in GC tissues, and E2F6 inversely regulated the expression of CASC2 in GC cells. More importantly, downregulation of E2F6 inhibited the proliferation and invasion and promoted the apoptosis of GC cells. However, these changes induced by E2F6 downregulation were reversed by CASC2 knockdown. Our findings suggest that E2F6 is a vital regulator of lncRNA expression and E2F6/CASC2 axis is a new mechanism for GC. In our future study, we will focus on the role of E2F6 and its molecular mechanism in chemotherapy and radiotherapy resistance of GC.

5. Conclusion

In summary, this study demonstrated that E2F6 was upregulated while CASC2 was downregulated in GC tissues and cell lines. Moreover, low expression of CASC2 was correlated with advanced TNM stage and worse survival in GC. Mechanically, E2F2 exerted its inhibitory role in GC progression by inhibiting the expression of CASC2. Our findings suggest that E2F6/CASC2 axis is another regulator of GC progression, which might facilitate the development of therapeutics against GC.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

None.

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