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*Original Article*

# Knockdown of LncRNA SBF2-AS1 Inhibited Gastric Cancer Tumorigenesis via the Wnt/LRP5 Signaling Pathway

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**This investigation aimed to uncover the impact of a long noncoding RNA, SET-binding factor 2 antisense RNA1 (SBF2-AS1) on the malignant progression of gastric cancer (GC) and to further explore its underlying mechanism. SBF2-AS1 expression was quantified by qRT-PCR in GC cell lines and GC tissues. *In vitro* loss-of-function studies of SBF2-AS1, accompanied by flow cytometry, CCK-8, and cell invasion tests, were applied to elucidate the impact of SBF2-AS1 on the tumor progression of GC cells. Finally, Western blotting and a luciferase assay were used to detect WNT/LRP5 signaling pathway activation. SBF2-AS1 was aberrantly expressed in GC cell lines ($p < 0.05$) and GC tissues ($p < 0.05$). Cell invasive and proliferative capabilities were inhibited via SBF2-AS1 knockdown, resulting in apoptosis of NCI-N87 and MKN74 cells. Additionally, online database analysis uncovered a positive correlation between SBF2-AS1 and the Wnt/LRP5 signaling pathway ($p < 0.05$). SBF2-AS1 knockdown blocked the Wnt/LRP5 signaling pathway, whereas the effects of SBF2-AS1 knockdown on the malignant genotype of MKN74 as well as NCI-N87 cells were partially restored by triggering the Wnt/LRP5 signaling pathway. High expression of SBF2-AS1 was found in GC, the malignant progression of which was repressed via SBF2-AS1 knockdown by inhibiting the Wnt/LRP5 signaling pathway.**

**Key words:** gastric cancer (GC), SET-binding factor 2 antisense RNA1 (SBF2-AS1), invasion, proliferation, signaling

Gastric cancers (GCs) are the third leading cause of cancer-related mortality worldwide [1]. On a global scale, and especially in East Asia, gastric cancer poses a major burden on health [2]. Despite the rapid development of medical techniques in recent decades, patients with GC are often diagnosed with distant metastases at an advanced stage, resulting in a relatively low 5-year survival rate [3,4]. Since specific clinical biomarkers are inadequate, patients with a variety of other tumors are diagnosed very late and therefore have reduced opportunities for survival; as a result, the 5-year survival rate is < 10%. There is thus is an urgent need for information regarding the mechanisms underlying GC carcinogenesis and for the development of new targeted treatment strategies.

The development and progression of GCs are complex biological processes involving a variety of genetic and epigenetic changes [5]. Along with the development of high-throughput sequencing technology, a multitude of long noncoding RNA (lncRNA) species

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that are longer than 200 nucleotides and that have no protein coding capacity have been identified in post-transcriptional modulatory processes [6]. There is increasing evidence that lncRNAs participate in the development and progression of malignancies through various regulatory mechanisms, such as RNA processing and stability, chromosomal remodeling, transcription, and/or post-transcriptional modulation [7]. However, few studies have examined the expression level and function of the SBF2-AS1 gene, a long noncoding RNA, in malignant tumors. It is worth noting that SBF2-AS1 is aberrantly expressed in glioblastoma and colorectal cancer and is a potential oncogene [8, 9]. Nevertheless, there has been no investigation into the expression patterns and function of SBF2-AS1 in GC.

## Materials and Methods

***GC patients and clinical specimens.*** From February 2012 to December 2014, GC tissues and corresponding para-cancerous tissue specimens were collected by tumorectomy from 78 patients (aged 37-76 years; 36 males and 42 females) at our hospital. Patients with other treatment pre-operation were not included in the investigation. Among the 78 GC patients, 31 had grade I-II and 47 had grade III-IV GCs. We obtained approval from the ethics committee of the Anhui cancer hospital, and all patients signed informed consent.

***Cell culture.*** Human GC cell lines (NCI-N87 and MKN74) were obtained from the Cell Bank of the Chinese Shanghai Academy of Sciences NCI-N87 and MKN74 cells were incubated in Dulbecco's Modified Eagle's Medium (Gibco, Waltham, MA, USA). GES-1 nonmalignant gastric mucosal epithelial cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in an incubator containing 5% $CO^2$ at 37°C.

***LncRNA array.*** Total RNA was extracted from GC and para-carcinoma control samples following the TRIzol® reagent protocol (Invitrogen, Carlsbad, CA, USA). Total RNA was first purified by an RNeasy mini kit (Qiagen, Valencia, CA, USA). The LncRNA expression profiles were examined using a human lncRNA expression array v3.0 (Arraystar, Rockville, MD, USA). After thoroughly cleaning the array, we scanned it and extracted the data by using Agilent feature extraction software.

***qRT-PCR.*** We extracted total RNA with TRIzol reagent and reverse-transcribed RNA into cDNA with a Reverse Transcriptase Kit (Promega, Madison, WI, USA). SBF2-AS1 expression levels were detected on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using an lncRNA qPCR assay kit (Tiangen, Beijing, China). The primers for SBF2-AS1 were F: 5'-AACCGCGGTTATGACA CCTTT-3' and R: 5'-CCAAGTATCCTGTCGCACA TCTC-3'. After the normalization of GAPDH, the $2-\Delta\Delta CT$ method was applied to quantitate the relative expression level of SBF2-AS1.

***Western blotting.*** We extracted proteins with a protein extraction kit (Life Technologies, Carlsbad, CA, USA). First, 30 μg protein was isolated with polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was then blocked with 5% nonfat milk at room temperature for 1 h. Afterwards, the membrane was hybridized with GSK3B, LRP5, and GAPDH antibodies (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. We then added goat anti-rabbit IgG secondary antibodies (1 : 4,000) followed by incubation at room temperature for 1 h. Finally, we analyzed the bands with ImageJ software (NIH, Bethesda, MD, USA).

***Transfection and luciferase assay.*** We designed and synthesized LRP5 expression plasmids (pEGFP-N1-LRP5, pE-LRP5) and purchased the silenced SBF2-AS1 expression plasmids (pGPU6/GFP/Neo-SBF2-AS1, sh-SBF2-AS1) from Gene Pharmacy (Shanghai, China). We used the blank plasmids pEGFP-N1 (pE-NC) and pGPU6/GFP/Neo (sh-NC) as negative controls. According to the protocol, we transfected the plasmids into NCI-N87 and MKN74 cells separately using Lipofectamine 3000 (Invitrogen). We co-transfected luciferase plasmids transiently into NCI-N87 and MKN74 cells for 48 h. We then quantified the luciferase reporters by a dual luciferase reporter gene assay kit (Beijing Biotech, Beijing, China).

***Cell proliferation assay.*** The proliferative abilities of NCI-N87 and MKN74 cells were determined by CCK-8 (Dojindo, Tokyo, Japan). We suspended and added cells into 96-well plates. In each well, cells were supplemented with 20 μL CCK-8 solution. The plates were cultured at 37°C for 2 h. Absorbance was detected at 490 nm ($OD_{490}$).

***Cell invasion test.*** The invasiveness of NCI-N87

and MKN74 cells was measured using a Matrigel matrix (Corning, Corning, NY, USA) chamber. Add culture medium containing serum to the lower chamber, and 100 μL DMEM was added to the upper chamber. Nci-n87 and MKN74 cells was then cultivated for 24 h at room temperature. We cleared the cells located within the upper chambers. Membranes were then fixed with methanol followed by staining with Giemsa solution. The invasive cells at 10 × magnification in three random fields were counted and the average value per field was calculated.

***Flow cytometry.*** An Annexin V/PI apoptosis detection kit (BioLegend, San Diego, CA, USA) was used to detect the apoptosis of MKN74 and NCI-N87 cells as described previously [10]. Generally, $1 \times 10^6$ cells were collected and cultivated with Annexin V/PI buffer. Apoptosis rates were analyzed by a FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

***Statistical analysis.*** SPSS software (v.22.0; IBM, Armonk, NY, USA) was used for the statistical analyses. Data were presented as means ± SD. Independent sample *t*-test and analysis of variance (ANOVA) were used for comparisons. Values of $p < 0.05$ were defined as statistically significant.

## Results

***SBF2-AS1 was overexpressed in GC cell lines and tissues.*** First, an lncRNA microarray analysis was conducted to identify lncRNAs that were differentially expressed between the GC and para-carcinoma control samples. The outcomes revealed that SBF2-AS1 was aberrantly expressed in GC samples relative to the control samples (Fig. 1A). qRT-PCR analysis further confirmed the overexpression of SBF2-AS1 in GC cell lines (NCI-N87 and MKN74) and specimens relative to control GES-1 cell lines and specimens (Fig. 1B-C, $p < 0.001$). These findings indicated that SBF2-AS1 was highly expressed, and the high expression of SBF2-AS1 may be associated with GC carcinogenesis as well as GC progression.

***The malignant phenotype of GC cells was inhibited by SBF2-AS1 knockdown.*** Whether or not the SBF2-AS1 knockdown has an impact on the malignant phenotype in GC was further assessed by loss-of-function studies. First, we transfected NCI-N87 and MKN74 cells with sh-SBF2-AS1 to inhibit SBF2-AS1 expression ($p < 0.01$, Fig. 2A). The results revealed that, compared to the sh-NC group, the proliferation and invasion of NCI-N87 and MKN74 cells were significantly inhibited by the SBF2-AS1 knockdown ($p < 0.01$, Fig. 2B-D).

***SBF2-AS1 knockdown inactivated the Wnt/LRP5 signaling pathway.*** The potential regulatory mechanism of SBF2-AS1 in GC cells was explored through an in-depth analysis of microarray data. The KEGG pathway detection revealed that the Wnt/LRP5 signaling pathway is the most abundant one. In addition, co-expression analysis of 163 GC samples in the TCGA Pan-Cancer database showed a significant negative correlation between SBF2-AS1 expression and GSK3b (Fig. 3A-B, $p < 0.01$, $r = -0.237$). Therefore, to highlight the SBF2-AS1–related GC malignant cell phenotype, we chose the Wnt/LRP5 signaling pathway as the target. Moreover, the SBF2-AS1 knockdown dramatically inhibited the TOP/FOP ratios of NCI-N87 and MKN74 cells, representing the transcriptional performance of the Wnt/LRP5 signaling pathway (Fig. 3C, $p < 0.01$). In addition, the SBF2-AS1 knockdown enhanced GSK3B protein expression and repressed LRP5 protein expression in both NCI-N87 and MKN74 cells (Fig. 3D, $p < 0.01$). In summary, the SBF2-AS1 knockdown inactivated the Wnt/LRP5 signaling pathway.

In addition, SBF2-AS1 knockdown inhibits the GC cell malignant phenotype through this pathway. We co-transfected PE-LRP5 into NCI-N87 and MKN74 cells with the SBF2-AS1 knockdown to activate the Wnt/LRP5 signaling pathway repressed by the knockdown. Wnt/LRP5 signaling pathway proteins were detected by Western blotting and the luciferase reporter assay (Fig. 4A-B, $p < 0.01$). Within the sh-SBF2-AS1 + pE-LRP5 group, the proliferation and invasiveness of NCI-N87 and MKN74 cells were significantly higher than those in the sh-SBF2-AS1 + pE-NC group ($p < 0.01$, Fig. 4C and 4D). Moreover, the apoptosis rate of the SH-SBF2-AS1 + PE-LRP5 group was remarkably lower than that of the SH-SBF2-AS1 + PE-NC group ($p < 0.01$, Fig. 4E). The proliferation and invasiveness of NCI-N87 and MKN74 cells were also significantly lower in the sh-SBF2-AS1 + sh-GSK3β group ($p < 0.01$, Fig. 5A-D). The apoptosis rate of the sh-SBF2-AS1 + sh-GSK3β group was remarkably higher ($p < 0.01$, Fig. 5E). It could be concluded that the activation of the Wnt/lrp5 pathway partially reversed the influence of SBF2-AS1 knockdown on the malignant phenotype in NCI-N87

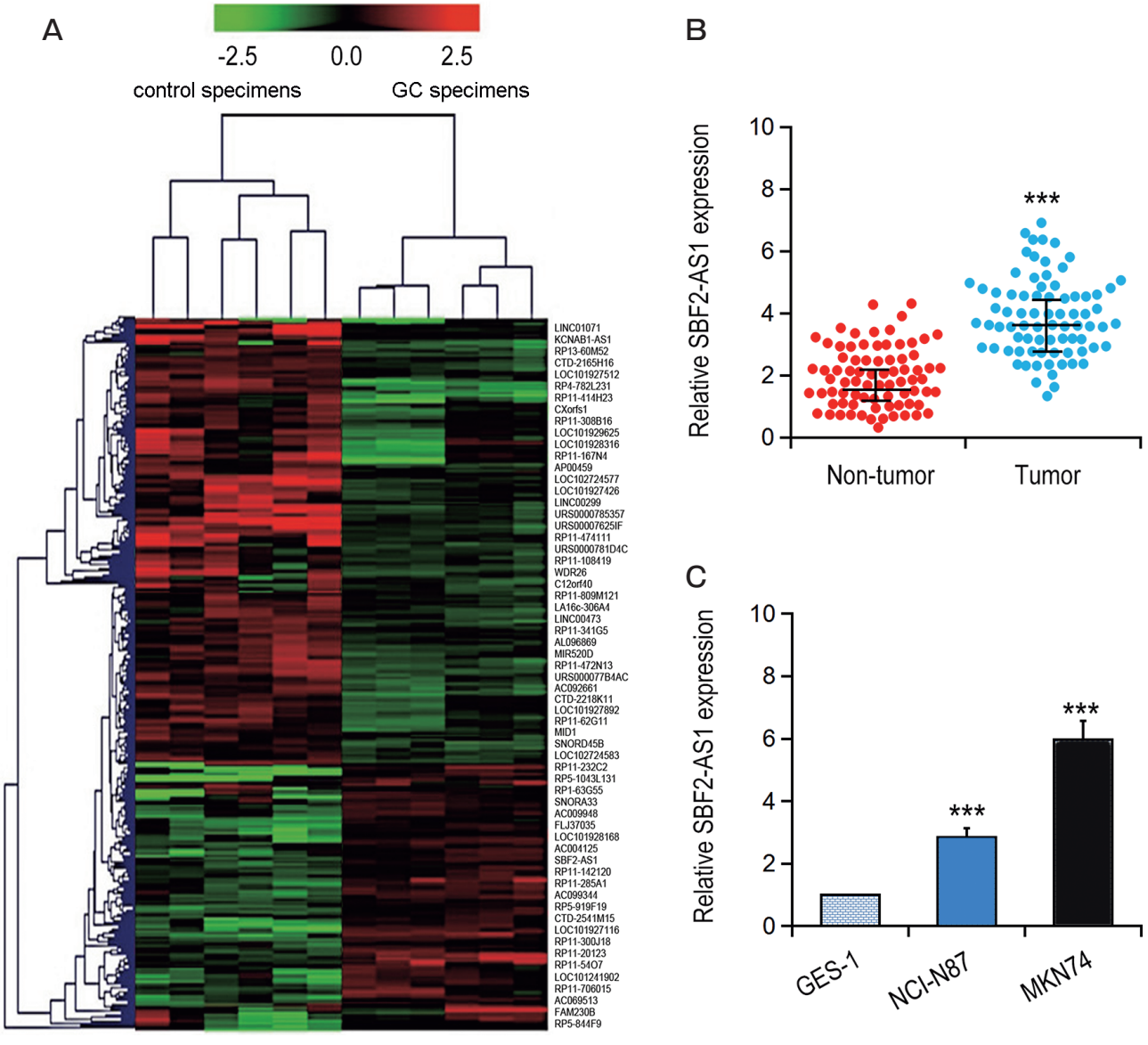


**Fig. 1** SBF2-AS1 was highly expressed in GC tissues and cell lines. **A**, Representative microarray analysis of SBF2-AS1 in GC and control specimens presented in a heatmap; **B**, The expression levels of SBF2-AS1 in GC specimens and control specimens. ***$p<0.01$ vs nontumor group; **C**, Expression levels of SBF2-AS1 in GES-1, NCI-N87, and MKN74 cell lines. ***$p<0.001$ vs GES-1 group.

and MKN74 cells. SBF2-AS1 knockdown inhibited the malignant phenotype of GC cells by inactivating the Wnt/lrp5 signaling pathway.

## Discussion

LncRNAs have shown to be involved in disease progression among various tumors and to be valuable diagnostic or clinical prognostic biomarkers, including in GC [11]. Although many lncRNAs have been verified and discovered in the human genome, only a very small portion have been validated experimentally or annotated functionally in GC [11-12]. Many studies have shown that lncRNA participates in tumor proliferation metastasis and recurrence, the epithelial-mesenchymal transition, and angiogenesis chemotherapy resistance [13]. For example, HOXA11-AS acts as a molecular sponge to promote tumorigenesis and angiogenesis of

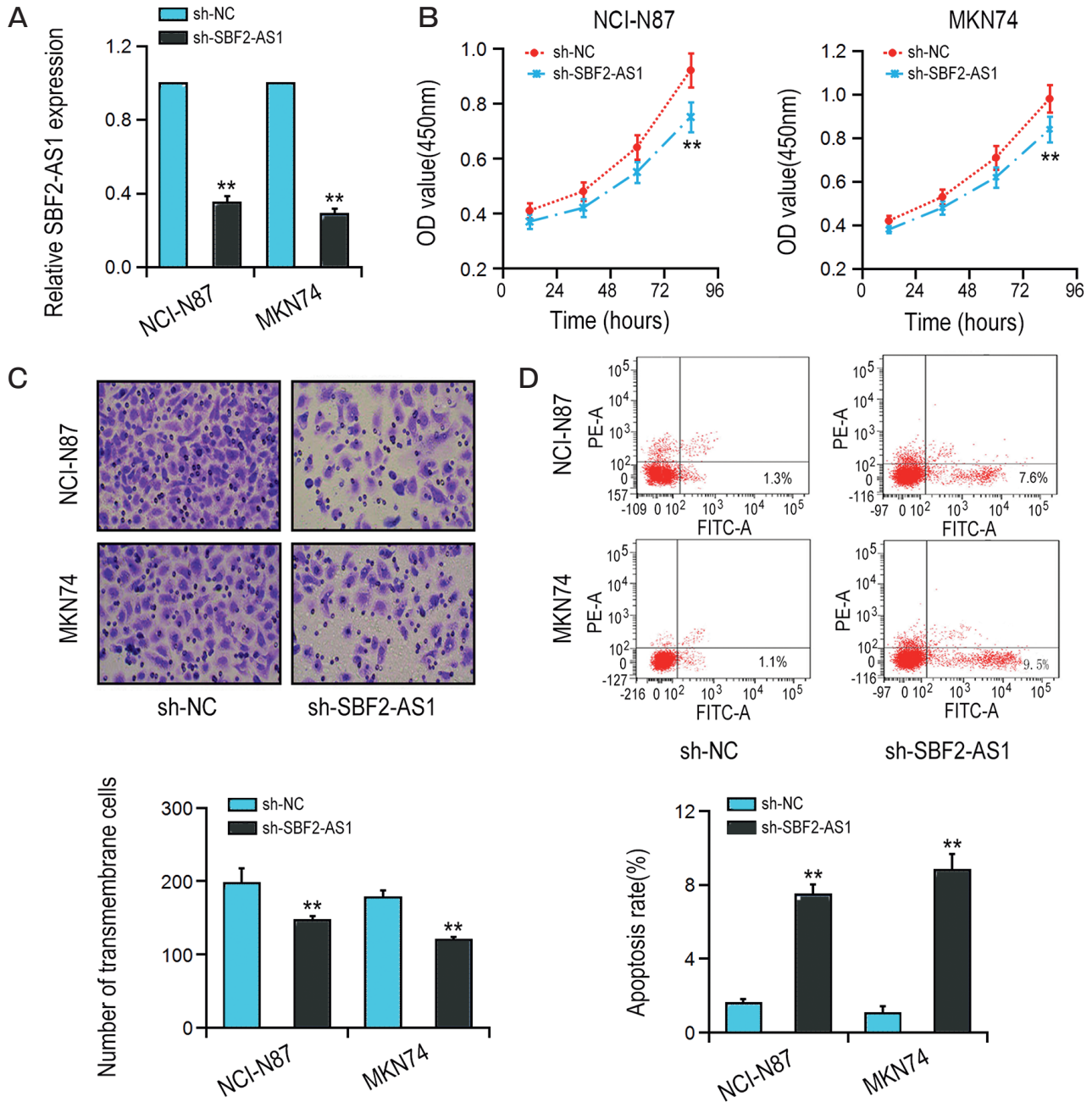


Fig. 2 Knockdown of SBF2-AS1 significantly inhibited malignant phenotypes of GC cells. **A**, Expression levels of SBF2-AS1 in NCI-N87 and MKN74 cells; **B**, Proliferation of NCI-N87 and MKN74 cells; **C**, Invasion of NCI-N87 and MKN74 cells; **D**, Cell apoptosis rates of NCI-N87 and MKN74 cells. $^{**}p<0.01$ vs sh-NC group.

the GC by targeting miR-1297 [14]. Chen *et al.* reported that VPS9D1-AS1 is a prognostic biomarker in GC patients and plays an anti-tumor role in hindering the malignant phenotype of GC cells [15].

SBF2-AS1 is known as the antisense strand of the SBF2-AS1 gene, which generally plays important regulatory roles in various human cancers. However, few reports have addressed the role of SBF2-AS1 in GC. The present lncRNA microarray analysis revealed for the first time that SBF2-AS1 expression is enhanced in GC,

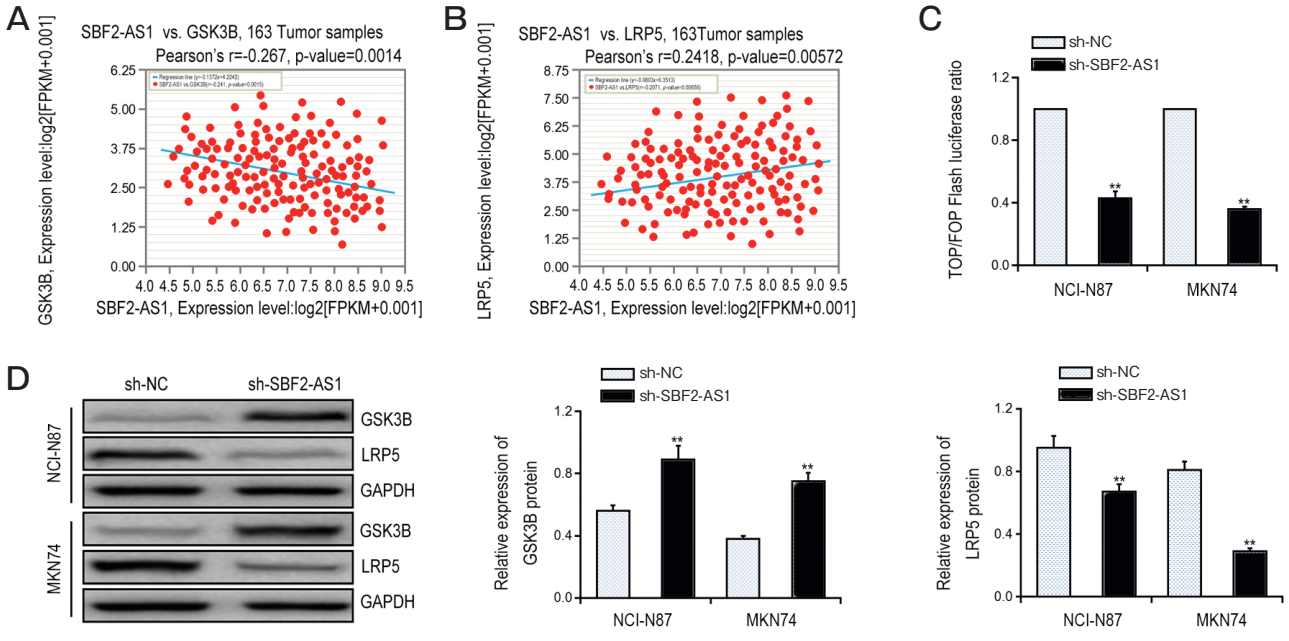


**Fig. 3** Knockdown of SBF2-AS1 inacti0vated the Wnt/LRP5 signaling pathway. **A**, Co-expression patterns between SBF2-AS1 and GSK3B in the TCGA Pan-Cancer database with 163 GC samples; **B**, Co-expression patterns between SBF2-AS1 and LRP5 in the TCGA Pan-Cancer database with 163 GC samples; **C**, Ratios of TOP/FOP luciferase values in NCI-N87 and MKN74 cells; **D**, Expression of GSK3B and LRP5 proteins in NCI-N87 and MKN74 cells. $^{**}p<0.01$ vs sh-NC group.

which was confirmed both in GC cell lines and GC samples, indicating that SBF2-AS1 might participate in GC tumorigenesis.

To verify the role of SBF2-AS1 in GC cells, loss-of-function studies were performed through SBF2-AS1 knockdown in GC cells. Recent studies have clarified the potential roles of SBF2-AS1 in non-small cell lung cancer and have revealed that high-level expression of SBF2-AS1 predicted a poor prognosis [16]. SBF2-AS1 was also verified to be highly expressed in colorectal cancer, suggesting that upregulation of SBF2-AS1 is a prognostic biomarker and potential therapeutic target of colorectal cancer [9]. Therefore, we conducted loss-of-function studies to determine whether SBF2-AS1 has an inhibitory role in GC. Our data showed that the proliferative and invasive capabilities of NCI-N87 and MKN74 cells were both greatly suppressed after SBF2-AS1 knockdown *in vitro* (both $p<0.05$). Those results suggested that knocking down SBF2-AS1 could limit the malignant phenotype of GC cells. However, the mechanism underlying this action is still unclear.

Many studies have demonstrated the essential role of the Wnt signaling pathway in cancer progression. The Wnt signal transduction pathway can regulate cell proliferation and differentiation as well as fate determination and serves as an evolutionarily conserved signaling pathway that modulates diverse cellular processes. Mutations in some key components, such as Axin, β-catenin, and APC, as well as in Wnt ligands, can modulate the progression of many carcinomas, including cerebral aneurysm, pancreatic ductal adenocarcinoma, lung cancer, mammary carcinoma, breast cancer, and gastrointestinal cancers. However, the mechanism underlying its activated expression in GC still needs to be elucidated. Through an in-depth analysis of the TCGA Pan-Cancer database and lncRNA microarray data, a positive regulation model of the SBF2-AS1 and Wnt/LRP5 signaling pathways was predicted, and this model was selected as a target to highlight the SBF2-AS1–related GC malignant phenotype. It is well known that the Wnt/LRP5 signaling pathway is frequently activated during tumorigenesis [17,18]. In our study, the results of Western blotting and the luciferase reporter assay confirmed that the SBF2-AS1 knockdown resulted in repression of this pathway. We therefore hypothesized that SBF2-AS1 knockdown significantly suppresses the malignant phenotype of GC cells by repressing the Wnt/LRP5 signaling pathway.

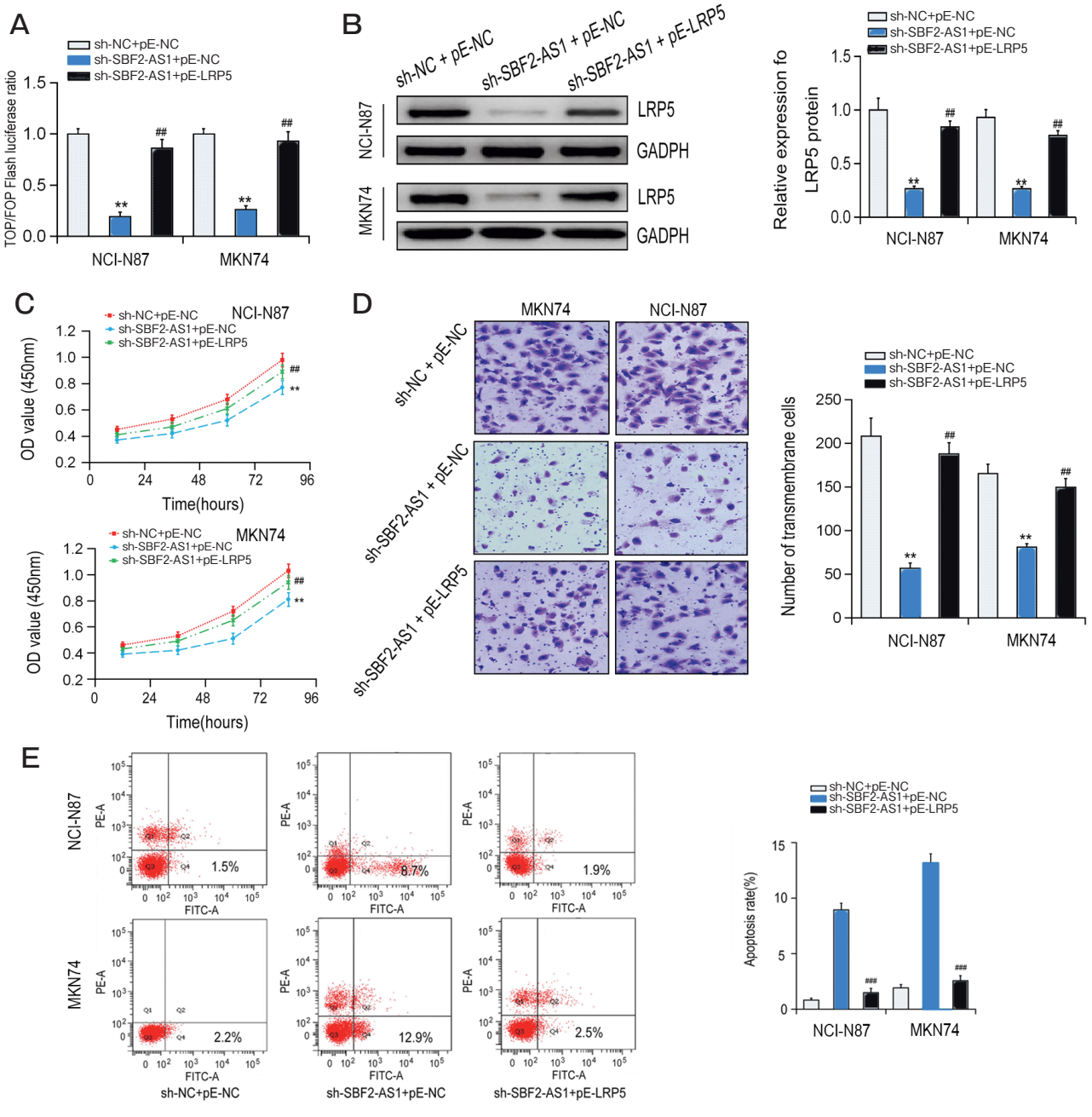


**Fig. 4** SBF2-AS1 knockdown greatly inhibited malignant phenotypes of GC cells via the Wnt/LRP5 signaling pathway. **A**, Ratios of luciferase values in NCI-N87 and MKN74 cells; **B**, Expression of LRP5 protein in NCI-N87 and MKN74 cells; **C**, Proliferation of NCI-N87 and MKN74 cells; **D**, Invasion of NCI-N87 and MKN74 cells; **E**, Apoptosis rates of NCI-N87 and MKN74 cells. $^{**}p<0.01$ vs sh-NC+pE-NC group, $^{\#\#\#}p<0.001$ vs sh-SBF2-AS1+pE-NC group.

Further experiments demonstrated that activation of the Wnt/LRP5 signaling pathway by LRP5 reversed the impact of the malignant phenotype of GC cells with SBF2-AS1 knockdown. The pathway mediated the effect of the knockdown on GC cells. We performed TOP/FOP Flash luciferase reporter investigations in both NCI-N87 and MKN74 cells, and our findings demonstrated that proliferation and invasiveness were restored

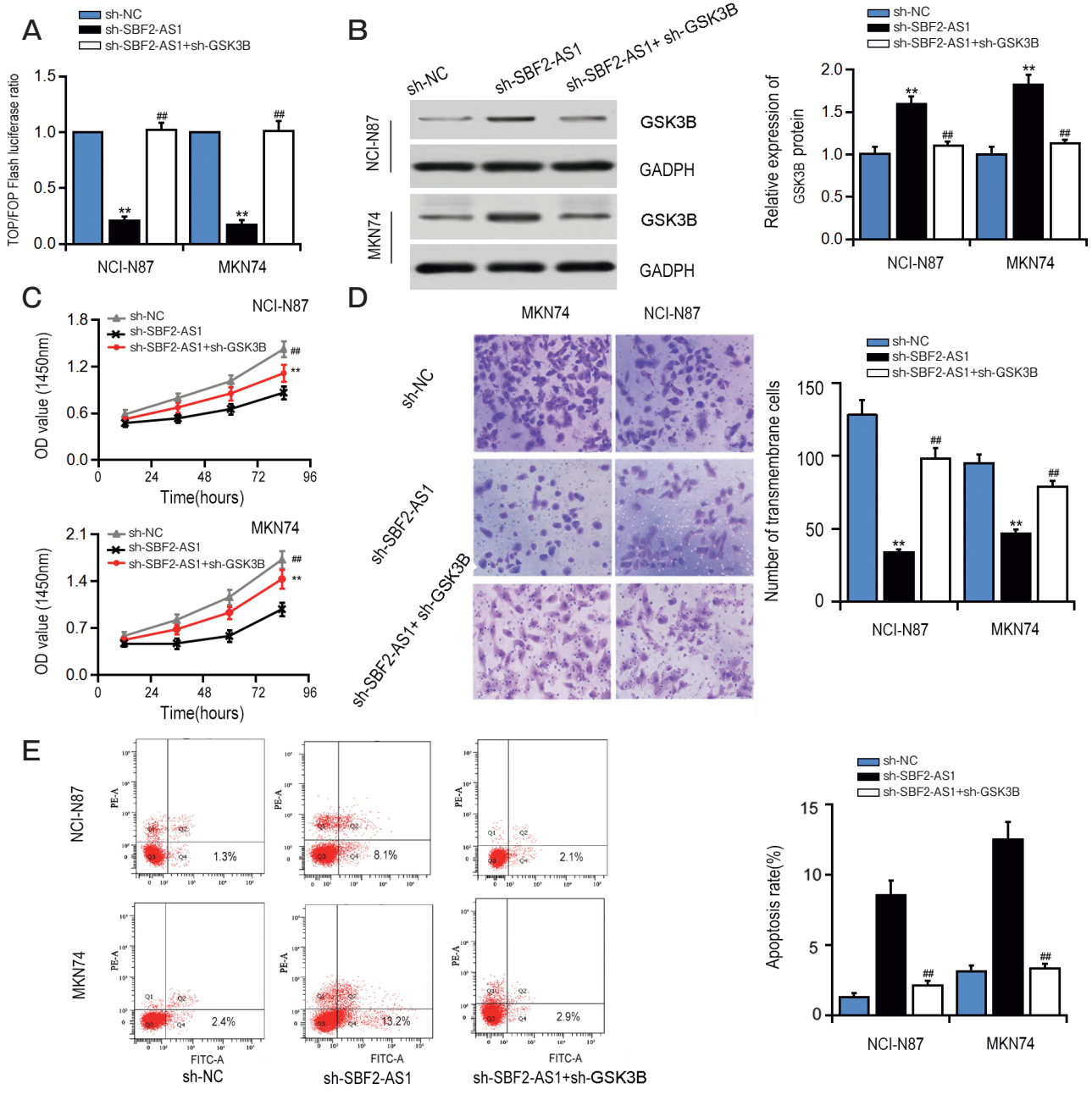


Fig. 5 The effects of sh-GSK3$\beta$ on cell proliferation, invasion, and apoptosis after SBF2-AS1 knockdown. **A**, Ratios of luciferase values in NCI-N87 and MKN74 cells; **B**, Expression of GSK3$\beta$ protein in NCI-N87 and MKN74 cells; **C**, Proliferation of NCI-N87 and MKN74 cells; **D**, Invasion of NCI-N87 and MKN74 cells; **E**, Apoptosis rates of NCI-N87 and MKN74 cells. $^{**}p<0.01$ vs sh-NC group, $^{\#\#\#}p<0.001$ vs sh-SBF2-AS1+sh-GSK3$\beta$ group.

after LRP5 overexpression in the SBF2-AS1 knockdown group ($p<0.01$). LRP5 overexpression also significantly reversed the increases in the apoptosis rate induced by SBF2-AS1 knockdown in comparison to the control group ($p<0.01$). Taken together, the above findings indicated that LRP5 was correlated with malignant progression in GC through the Wnt/LRP5 signaling pathway. A previous study demonstrated that SP1-modulated

miR-545-3p functions as an osteogenesis-inhibitory factor by targeting LRP5 to inactivate Wnt/β-catenin signaling [19], while another study showed that inhibition of miR-545 or overexpression of EMS1 partially reversed the SBF2-AS1-depletion-induced suppression of proliferation, migration, and invasion [20]. These results were consistent with the present results.

However, there are still limitations to our study that need to be addressed in our further investigations. For example, we did not investigate whether SBF2-AS1 regulates the Wnt/LRP5 signaling pathway as we hypothesized, or whether there are any other possible pathways that may be associated with the above-mentioned modulatory way. Finally, the relationship between cancer grade and the expression level of SBF2-AS1 was not evaluated, and the KEGG pathway data were not considered in this study.

In conclusion, SBF2-AS1 expression is upregulated in GC, and this upregulation positively regulates the malignant phenotype of GC through the Wnt/LRP5 signaling pathway. The present results will be of great value for elucidating the molecular mechanisms underlying GC carcinogenesis. Such elucidation could offer an alternative strategy for GC therapy targeting LncRNA.

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