Neoplasma 2024; 71(3): 209–218

doi:10.4149/neo_2024_240220N67

# Downregulation of WNK4 expression facilitates the proliferation of gastric cancer cells via activation of the STAT3 signaling pathway

Miao LI[1,#], Xiaoyan SHAO[2,#], Qiqi NING[3], Rongrong SUN[2], Rantian LI[2], Yanhua LIU[2], Yuan YUAN[2,*], Youwei ZHANG[1,2,*]

[1]*Jinzhou Medical University Postgraduate Training Base (Xuzhou Central Hospital), Jinzhou, China;* [2]*Department of Medical Oncology, Xuzhou Central Hospital, Xuzhou, China;* [3]*Department of Molecular Microbiology and Immunology, University of Southern California Keck School of Medicine, Los Angeles, California, United States*

**Correspondence: yuanyuan87303@163.com, zhangyw@njmu.edu.cn*

**Received February 20, 2024 / Accepted May 1, 2024**

WNK lysine deficient protein kinase 4 (WNK4) has been shown to be significantly associated with cancer progression. Nevertheless, its involvement in gastric cancer (GC) is unclear. The objective of this work was to investigate the WNK4's regulatory mechanism in GC. Quantitative RT-PCR and immunoblots revealed that WNK4 expression was downregulated in GC and that low expression of WNK4 was strongly linked to poor prognosis. Functional assays including cell counting kit-8 assay and colony formation assay demonstrated that overexpression of WNK4 led to limited tumor proliferation both *in vitro* and *in vivo*, while the WNK4 reduction yielded to the opposite results. Gene Set Enrichment Analysis (GSEA) indicated a potential association between WNK4 and the signal transducer and activator of transcription (STAT3). WNK4 suppressed the phosphorylation of signal transducer and activator of transcription 3 (p-STAT3) in GC cells. The inhibition of the STAT3 pathway with Stattic reversed growth and proliferation induced by WNK4 knockdown in GC cells. These findings provide new insights for identifying key therapeutic targets for GC in the future.

*Key words: WNK4; gastric cancer; proliferation; tumorigenesis; STAT3*

According to the latest GLOBOCAN 2020 data, gastric cancer (GC) ranked fifth globally in terms of incidence of malignant tumors [1]. GC patients were typically diagnosed at intermediate or advanced stages, resulting in missed opportunities for optimal surgical intervention and unfavorable prognoses. Despite the availability of comprehensive treatment approaches such as palliative surgery, radiotherapy, chemotherapy, etc., their efficacy remained unsatisfactory [2–4]. Particularly when compared to the progress made in targeted therapy for advanced lung adenocarcinoma cases over recent years, patients with advanced GC experienced a lagging prognosis. This can be primarily attributed to the high heterogeneity of GC and its yet incompletely understood pathogenesis. Although targeted therapy and immunotherapy had introduced new strategies for treating GC, overall survival rates for advanced cases had not significantly improved [5, 6]. Therefore, elucidating the mechanisms behind GC is crucial for optimizing treatment regimens.

STAT3 is a multifunctional molecule engaged in signal transduction and transcriptional activation, playing crucial roles in inflammation, metabolism, and tumorigenesis [7, 8]. Previous studies have shown that the IL-6/IL-8/IL-11-mediated JAK-STAT pathway promotes GC growth and invasion [9–11]. Additionally, *Helicobacter pylori* (*H. pylori*) infection activated STAT3, which binds directly to the TMEFF2 gene promoter region leading to its downregulation. Simultaneously, TMEFF2 negatively regulated STAT3 activation through SHP-1, thereby promoting GC progression through this feedback loop [12]. Moreover, SPI1 enhanced the malignant phenotype of GC through activating the IL-6/JAK2/STAT3 signaling pathway [13]. Targeting the STAT3 pathway holds significant potential for innovative treatment strategies against GC. Costa et al. [14] discovered that the silencing of the WNK2 kinase activates c-Jun N-terminal kinase (JNK), thus promoting glioma development. Given the close association between JNK kinase and the STAT3 signaling pathway, it was presumed that WNK2 acted via activating the STAT3 pathway. Consequently, WNK might played a pivotal role in tumorigenesis.

With-no-lysine (WNK) kinases, a class of serine/threonine protein kinases, were implicated in various cancer-related signaling cascades, highlighting their multifaceted

roles [15, 16]. WNK1 is implicated in adverse prognosis in hepatocellular carcinoma (HCC) and colorectal cancer (CRC) [17, 18]. Conversely, silencing of WNK2 correlated with tumor recurrence and inferior overall survival (OS) in HCC, accompanied by invasion of tumor-associated macrophages [19]. WNK3 potentiates invasiveness in a manner that induces glioma epithelial-to-mesenchymal transition in hypoxic conditions [20]. Upregulation of WNK4 enhanced medulloblastoma cell proliferation and confers resistance to cisplatin treatment, contributing to poor prognosis [21]. Nevertheless, WNK4's involvement in GC remains to be explored.

We investigated the influence and manifestation of WNK4 in GC cells and noted that WNK4 operates as a cancer inhibitor in GC. Moreover, downregulation of WNK4 expression augmented STAT3 pathway activity in GC cells, while inhibition of the STAT3 pathway with Stattic reversed growth and proliferation induced by WNK4 knockdown in GC cells. These findings suggested that reduced WNK4 expression promoted the growth of GC cells through activation of the STAT3 signaling pathway, thereby providing a promising direction for GC therapy.

## Materials and methods

**Data collection and analysis.** In previous work, one hundred GC tissues and paired normal tissue specimens were retrieved from our specimen bank of Xuzhou Central Hospital were mobilized to detect mutations in the WNK family genes by targeted sequencing. It was carried out according to the ethical standards approved by the Ethics Committee of the hospital. To examine the level of WNK4 in GC, we utilized the Box plot function available in the Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/index.html). Furthermore, the University of ALabama at Birmingham CANcer (UALCAN) database platform (http://ualcan.path.uab.edu/index.html) was utilized to investigate changes in gene expression across various grades and stages of GC. The immunohistochemistry findings from Human Protein Atlas (HPA) database (https://www.proteinatlas.org) provided valuable insights into the expression patterns of WNK4 in various tissues. To explore the prediction value of WNK4 expression on GC survival, we downloaded the normalized mRNA expression profiles of GC cohort (N = 410) and its corresponding survival information from https://xena.ucsc.edu/. To carry out survival analysis, the 'survminer' package was applied. In order to identify the most significant survival differences, the surv_cutpoint function, with a minprop value of 0.1, was utilized to determine the optimal threshold for grouping.

**Cell lines and cell culture.** For this study, we obtained GC cell lines (AGS, HGC27, MKN28, MKN45, and NCIN87) and the human gastric epithelium cell line (GES-1) from the Cell Resource Center at the Shanghai Institutes for Biological Sciences, Chinese Academy. Culturing of the cells involved the RPMI1640 (Biosharp, #BL303A) supplemented with 10% fetal bovine serum (Gibco, #16000-044) and 1% penicillin/streptomycin (Solarbio, #P1400-100). The cells were incubated at 37 °C with a $CO_2$ concentration of 5% to maintain the optimal growth conditions.

**Lentivirus infection.** Overexpressing and shRNA lentiviral WNK4 vectors were constructed by Shanghai GenePharma Co (China). shRNAs for WNK4-1, WNK4-2, or WNK4-3 were inserted into the pLKO.1 plasmid (Supplementary Table S1). To overexpress WNK4, coding sequences were inserted into the pLVX-Puro plasmids. AGS or MKN45 cells were seeded individually during their logarithmic growth phase at a density of $5\times10^5$ cells/well into the 6-well culture plates. Following this, the cells were lentivirally transfected until reaching approximately 60% confluency. To carry out subsequent experiments, the cells were collected after 72 h by adding a blend of 5 μl lentivirus and 1 μl polybrene (5 μg/μl) to each well.

**Western blotting analysis.** Protein extraction was conducted using the rapid lysate cell kit (Beyotime, #P0013). The protein concentration was determined with the BCA Protein Assay kit (Beyotime, #P0012). The protein lysate separated by 10% SDS-PAGE was transferred onto the NC membrane (Millipore, #HATF00010). We blocked the membranes with 5% skimmed milk. Subsequently, they were incubated overnight at 4 °C with following primary antibodies: WNK4 (1:1000, Cell Signaling Technology (CST), #5713), STAT3 (1:1000, Abcam, #AB68153), p-STAT3 (1:1000, Abcam, #AB267373), GAPDH (1:2000, CST, #5174). The blots were further treated with an additional incubation period for 1 h with HRP-labelled anti-mouse (1:1000, Beyotime, #A0216) or anti-rabbit (1:1000, Beyotime, #A0208). Finally, enhanced visualization of target protein bands.

**Cell counting Kit-8 (CCK-8) assay.** In order to evaluate cell proliferation, we utilized the CCK-8 assay. In concrete terms, cells were cultured in 96-well plates with an initial density of $3\times10^3$ cells/well at 37 °C in a 5% $CO_2$ environment for an overnight duration. Subsequently, the supernatant was removed, and each well received 10 μl of CCK-8 reagent (Signalway Antibody (SAB), #CP002) at 0, 12, 24, 48, and 72 h. Incubation of the plates was then carried out under light protection for one hour. Eventually, the absorbance at 450 nm was conducted.

**Colony formation assay.** $1\times10^3$ cells/well were inoculated in a culture plate routinely. The plates were stored in an incubator at 37 °C for a total duration of 21 days. The removed liquid above the cells was eliminated, and the cells were rinsed twice. Cells were fixed using a solution of 4% paraformaldehyde for a period of 15 min. Afterward, staining was applied with 0.1% crystal violet solution for 10 min. Lastly, the staining solution was slowly washed off with running water, and the plates were left to dry at room temperature before observing the colonies.

**Real-time quantitative PCR (qPCR).** Extraction of total RNA was carried out with TRIzol reagent (Invitrogen, #1596-026). Following this, a reverse transcription kit (Yesen, #11123ES60) was used for synthesizing cDNAs. The SYBR Green PCR kit (Yesen, #11202ES03/08/60) was utilized for conducting real-time quantitative PCR analysis. A PCR detector (Heal Force) was employed under specific conditions: a denaturation step, lasting for 5 min at 95 °C, which was followed by a series of 40 cycles. Each cycle comprises 10 s of denaturation and 30 s of annealing/extension at the corresponding temperature. Each reaction consisted of 10 ng of sample cDNA and HPLC-grade water to make up a final volume of 20 µl. GAPDH served as an internal control to ensure accuracy. The $2^{-\Delta\Delta CT}$ approach was employed for the computation of the relative expression of WNK4. The primer sequences utilized are presented in Table 1.

**Nude mice xenograft model.** We obtained four- to six-week-old male BALB/c-nude mice from the Xuzhou Medical University Laboratory Animal Center. They were randomly allocated into the oe-WNK4 group and Vector group (n=6/group). Nude mouse xenograft models were established by subcutaneously injecting 100 µl ($5\times10^6$ cells) of AGS cells transfected with either the WNK4 vector or the empty vector. During our study, tumor growth indicators, including the measurements of tumor length (L) and width (W), were assessed at intervals of three days. Tumor volume was calculated as $½ \times (L \times W2)$. After 5 weeks, euthanasia of the mice was performed through cervical dislocation. The resulting tumors were excised, weighed, and subsequently preserved at –80 °C. All of the animal experimental protocols received approval from the Experimental Animal Ethics Committee of Xuzhou Medical University.

**Immunofluorescence (IF) staining.** The subcutaneous tumor tissues obtained from nude mice were fixed and embedded for dehydration. Afterward, the specimens were sectioned into a thickness of 5 µm and mounted on glass slides. The sections underwent an overnight incubation at 4 °C with a rabbit monoclonal antibody against Ki-67 (dilution of 1:200; Abcam; #AB243878). Subsequently, they were treated with Alexa Fluor488-conjugated IgG (dilution of 1:500; Beyotime; #A0423) at a temperature of 25 °C for 1 h. DAPI staining was performed followed by image acquisition with confocal microscopy. Fluorescence intensity was finally analyzed by using Image J.

**Gene Set Enrichment Analysis (GSEA).** To perform gene set enrichment analysis, we selected GC with the highest expression of WNK4 (>Q3) or the lowest expression of WNK4 (<Q1) from TCGA database to identify the signaling pathways primarily linked to WNK4 expression. Using the MSigDB hallmarked gene set collection, GSEA was conducted through the JAVA program available at (http://software.broadinstitute.org/gsea/index.jsp). This analysis involved ranking the genes based on their enrichment score, ranging from the most positive to the most negative. By doing so, it allowed us to assess the general correlation between gene sets and WNK4 expression. We conducted 1,000 arbitrary permutations on a sample and set the significance threshold for the p-value of the Normal distribution to be <0.05. If a cluster of genes exhibited a positive enrichment score, most of its constituents displayed elevated expression levels in conjunction with decreased WNK4 expression, therefore labeling the cluster as "enriched".

**Table 1. Primer sequences for real-time qPCR.**

| Genes | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| WNK4 | TCGGATTGCGAGACTGATG3 | GAACGTGGAATGGATAGGG |
| GAPDH | CGGATTTGGTCGTATTGG | CTCGCTCCTGGAAGATGG |

**Statistical analysis.** Mean ± SD of three or more independent experiments was employed for the presentation of all data after performing statistical analysis through SPSS 19.0 and GraphPad Prism 9.0. To compare gene expression among samples at various stages and grades, we utilized the Kruskal-Walli's test, whereas a one-way ANOVA test was employed to perform multiple comparisons. The correlations from WNK4 to genes within the JAK-STAT pathway were computed by the Pearson Correlation Coefficient. The notable differences between the two groups were conducted by Student's t-test. A p-value <0.05 was considered statistically significant.

## Results

**WNK4 was downregulated in GC and associated with a poor prognosis.** Initially, we performed targeted sequencing of one hundred gastric cancer tissues and paired normal tissue specimens in order to detect mutations of WNK family genes in gastric cancer, and found that WNK1, WNK2, WNK3, and WNK4 were mutated in gastric cancer tissues, with WNK4 having a significantly higher mutation rate of 8% compared with other members (Figure 1A). Therefore, we focused on the relationship between WNK4 and gastric cancer. We employed an online application based on analysis of TCGA database to compare mRNA levels of WNK4 between GCs and normal tissues. Our findings revealed a statistically significant downregulation of WNK4 mRNA expression in GC tissue (Supplementary Figure S1A). We characterized WNK4 protein expression in the HPA database.

**Immunohistochemical analysis with antibody HPA016500.** Remarkably, reduced levels of WNK4 were observed in GC tissues compared to moderate to high levels found in normal gastric tissue samples (Supplementary Figure S1B). There was a gradual decrease in WNK4 mRNA levels with increasing grade of GC (Supplementary Figure S1C). Although no noticeable variances were observed among different stages of GC (Stage I–IV), there was a trend towards reduced WNK4 mRNA expression as the disease progressed (Supplementary Figure S1D). In addition, we validated the results of bioinformatics analysis through WB and qPCR experiments. The data revealed that WNK4 exhibited significantly lower mRNA expression levels (Figure 1B)

and protein levels (Figure 1C) in GC cell lines compared to the GES-1 cell line. TCGA standardized GC cohort (N=410) mRNA expression patterns and their corresponding survival information were downloaded to investigate the predictive value of WNK4 expression for GC survival. Patients with an elevated WNK4 expression had a prolonged OS, as confirmed by univariate survival analysis (Supplementary Figure S1E). Combined, the above results suggest that WNK4 affected the progression and prognosis of GC.

**Overexpression of WNK4 in GC cells inhibited proliferation *in vitro* and *in vivo*.** Among the five GC cell lines, the AGS cell line was the one with the lowest endogenous WNK4 expression. We then investigated the impact of WNK4 on GC cell biology behavior by transfecting AGS cells with WNK4 slow retroviruses (AGS/oe-WNK4). The results obtained from RT-PCR and WB analysis demonstrated a high transfection efficiency (Figures 2A, 2B). Besides, it was observed that overexpression of WNK4 notably restrained the proliferation and colony-forming ability of AGS cells through CCK-8 assay and colony-forming assay (Figures 2C, 2D). To further validate these findings, we established an *in vivo* subcutaneous xenograft model using either AGS/oe-WNK4 or normal control AGS cells (Vector), which unequivocally confirmed that WNK4 overexpression effectively inhibited the tumorigenic potential of AGS cells (Figures 2E, 2G). Furthermore, immunofluorescence experiments conducted on paraffin sections showed a remarkable reduction in the Ki-67 index of subcutaneous tumors formed by AGS/oe-WNK4 cells compared to those formed by AGS/Vector cells as depicted in Figures 2H and 2I. Thus, our study provided compelling evidence supporting the inhibitory role of WNK4 in GC.

**Knockdown of WNK4 resulted in GC cell progression.** WNK4 expression was greatest in MKN45 cells among the five GC cell lines, thereby we downregulated WNK4 expression in the MKN45 cell line. First, the efficiency of knockdown was assessed by utilizing RT-PCR and WB techniques. (Figures 3A, 3B). Subsequently, shWNK4-1 and shWNK4-2 exhibiting superior transfection efficiency were chosen for further experiments. As anticipated, WNK4 knockdown did result in the promotion of cell proliferation and colony formation (Figures 3C, 3D).

**The close association between WNK4 expression and the JAK-STAT3 signaling pathway.** WNK4 acted as a tumor suppressor gene in GC through the aforementioned experimental results. Based on the gene expression profiles of GC

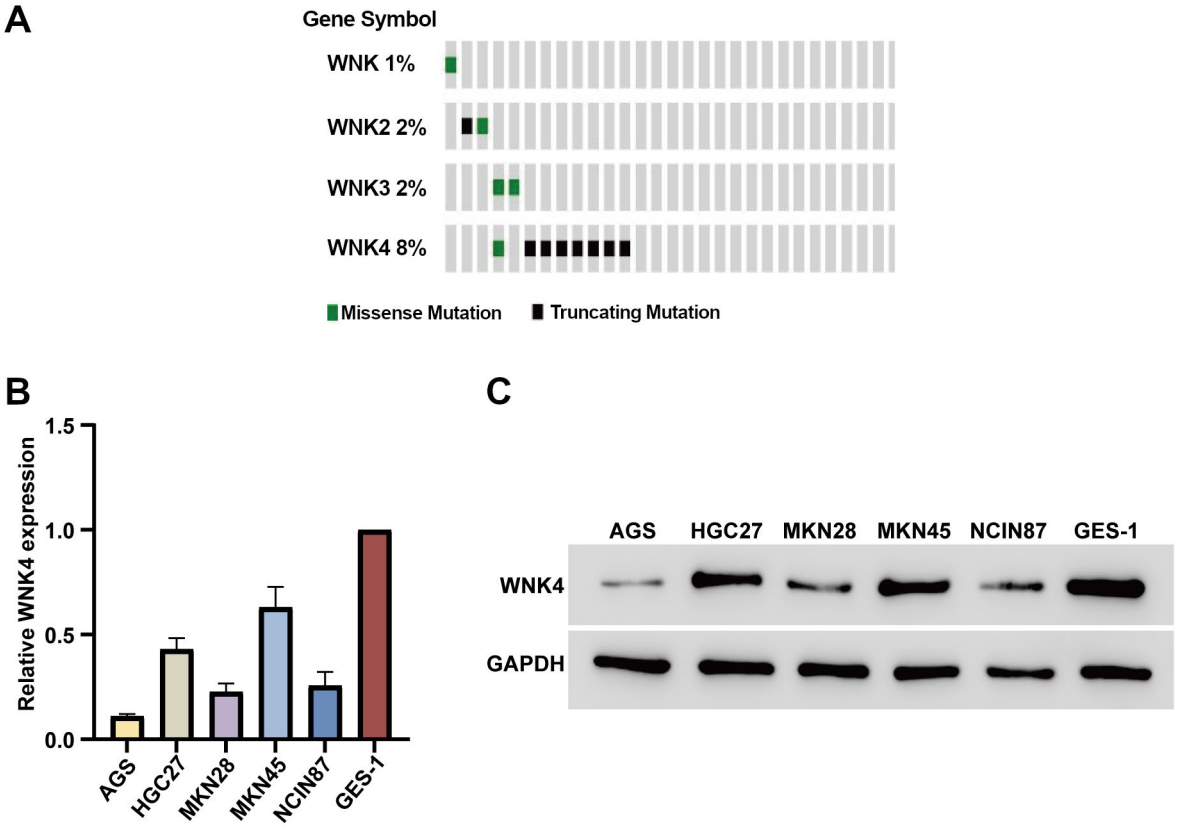



**Figure 1. WNK4 was downregulated in GC cell lines. A) Targeted sequencing results of 100 pairs of GC samples. Gray rectangles signify different exons, missense mutations (green), and truncation mutations (black) all localized in specific exons. B, C) The expression level of WNK4 in five GC cell lines (AGS, HGC27, MKN28, MKN45, NCIN87) and a normal gastric mucosal cell line (GES-1).**

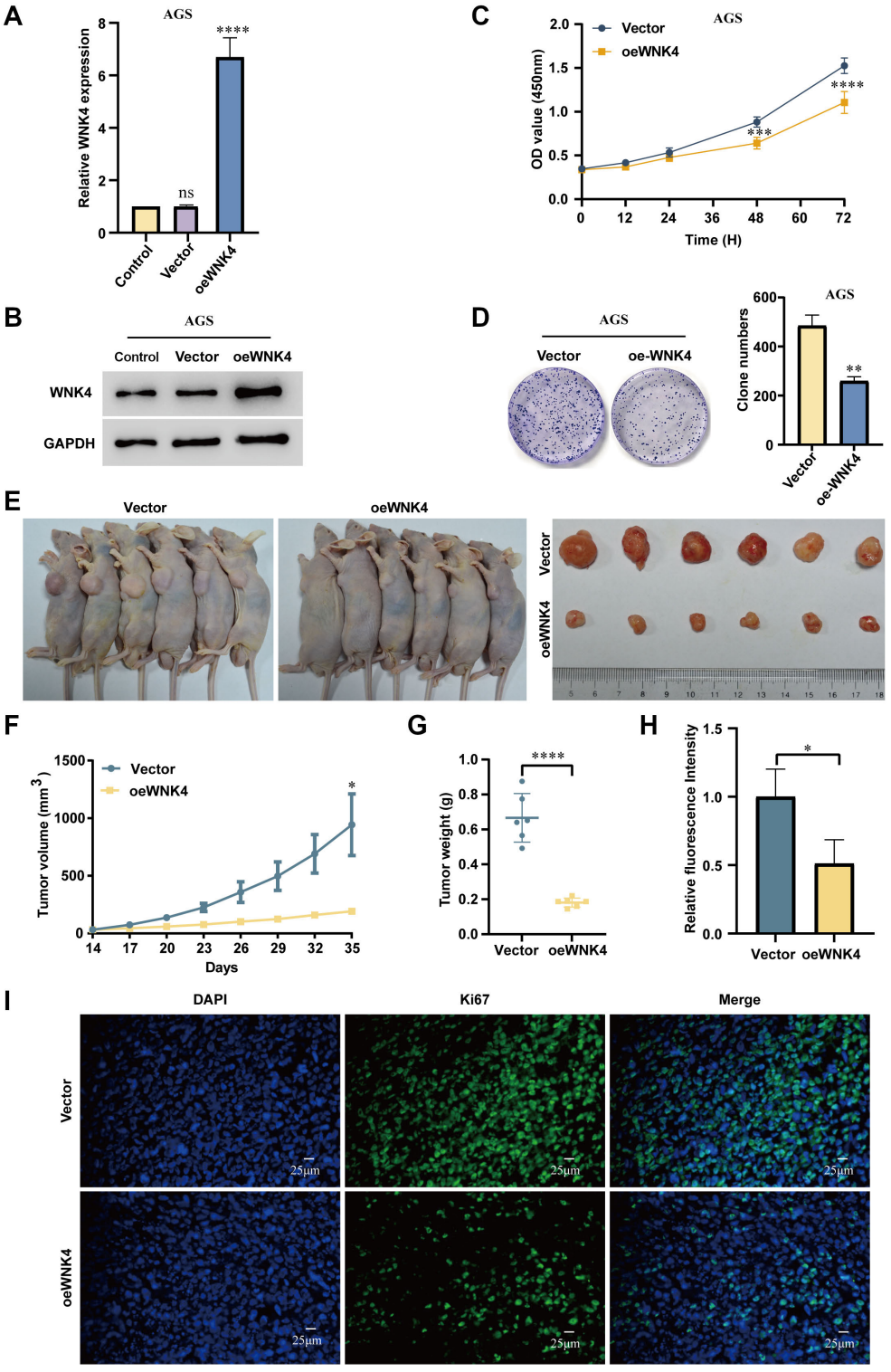



**Figure 2. WNK4 overexpression inhibited GC cells' proliferation *in vitro* and *in vivo*. A) RT-PCR and B) WB indicated the overexpression efficiency of WNK4 in AGS cells. C) Overexpression of WNK4 effectively inhibited AGS cells' proliferation as detected by CCK-8 assay. D) Colony formation assay revealed that overexpression of WNK4 inhibited the growth of AGS cell lines. E) Subcutaneous graft tumors formed by AGS/oe-WNK4 cells in nude mice were smaller than those generated by controls. F, G) Tumor growth curves and final tumor weights were revealed, respectively, and both metrics had the same trend. H, I) Two groups of tumor specimens were subjected to immunohistochemical detection of Ki-67 indicators (magnification ×400). Ki-67 (green) was assessed by immunofluorescence staining. DAPI was used for nuclear staining (blue). Data were expressed as the mean ± SD; *p<0.05, **p<0.01, ****p<0.0001**

extracted from TCGA database, we applied GSEA to explore the potential signaling pathways associated with WNK4 expression. The results showed a significant association between WNK4 and the IL-6/JAK/STAT3 signaling pathway, indicating that low WNK4 expression could lead to activation of the IL-6/JAK/STAT3 signaling pathway (NES=1.7, p=0.0077, Figure 4A). Furthermore, we extracted the key genes in the JAK-STAT3 pathway from the KEGG database and calculated the expression correlation with WNK4. The results showed that most key genes in the JAK-STAT3 pathway showed a significant negative correlation with WNK, including JAK1/JAK2/JAK3, STAT1/STAT2/STAT3/STAT4/STAT5A (Figure 4B). These results indicated the close association between WNK4 expression and the JAK-STAT3 signaling pathway.

**Knockdown of WNK4 activated the STAT3 signaling pathway in GC cells.** STAT3 is a multifunctional molecule involved in signal transduction and transcriptional activation in biological processes including inflammation, metabolism, and tumorigenesis [7, 8]. STAT 3 signaling pathway has been previously demonstrated in GC [22–25]. We revealed the correlation of WNK4 with the STAT3 signaling pathway in GC for the first time. Firstly, we examined the activation of STAT3 (tyrosine phosphorylation at Y705), as depicted in Figure 5A. AGS cells overexpressing WNK4 exhibited a significant reduction in p-STAT3 (Y705) levels, while MKN45 cells with knockdown of WNK4 showed a substantial increase in p-STAT3 (Y705) expression (Figure 5B). Subsequently, we employed Stattic – a non-peptide small molecule known to selectively inhibit STAT3 SH2 domain

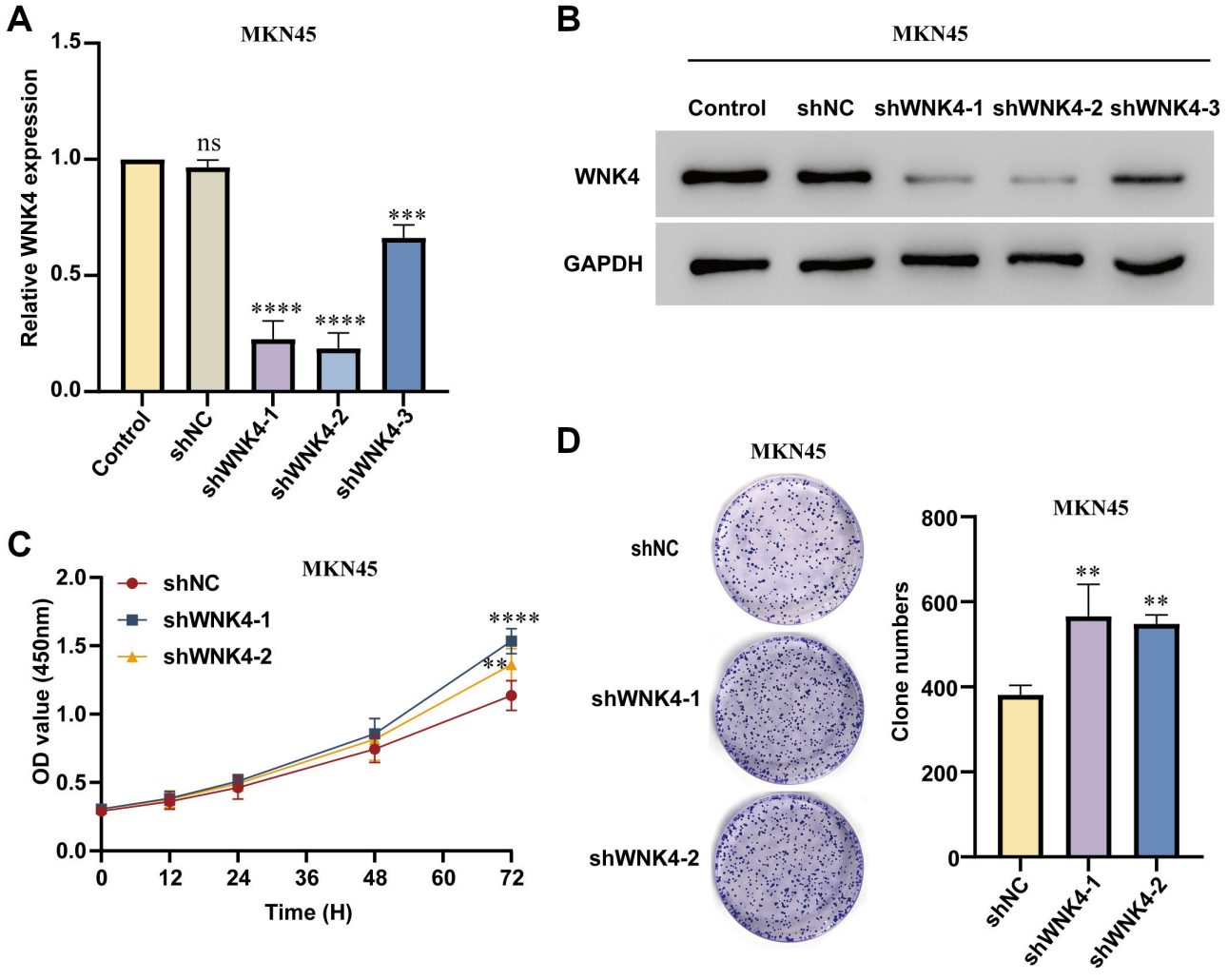



**Figure 3. Knockdown of WNK4 induced GC progression. (A) RT-PCR and (B) WB indicated knock-down efficiency of WNK4 in MKN45 cells. Knockdown of WNK4 promoted MKN45 cells' proliferation as detected by CCK-8 assay (C) and colony formation assay (D). Data were expressed as the mean ± SD; ** p<0.01, ***p<0.001, ****p<0.0001**

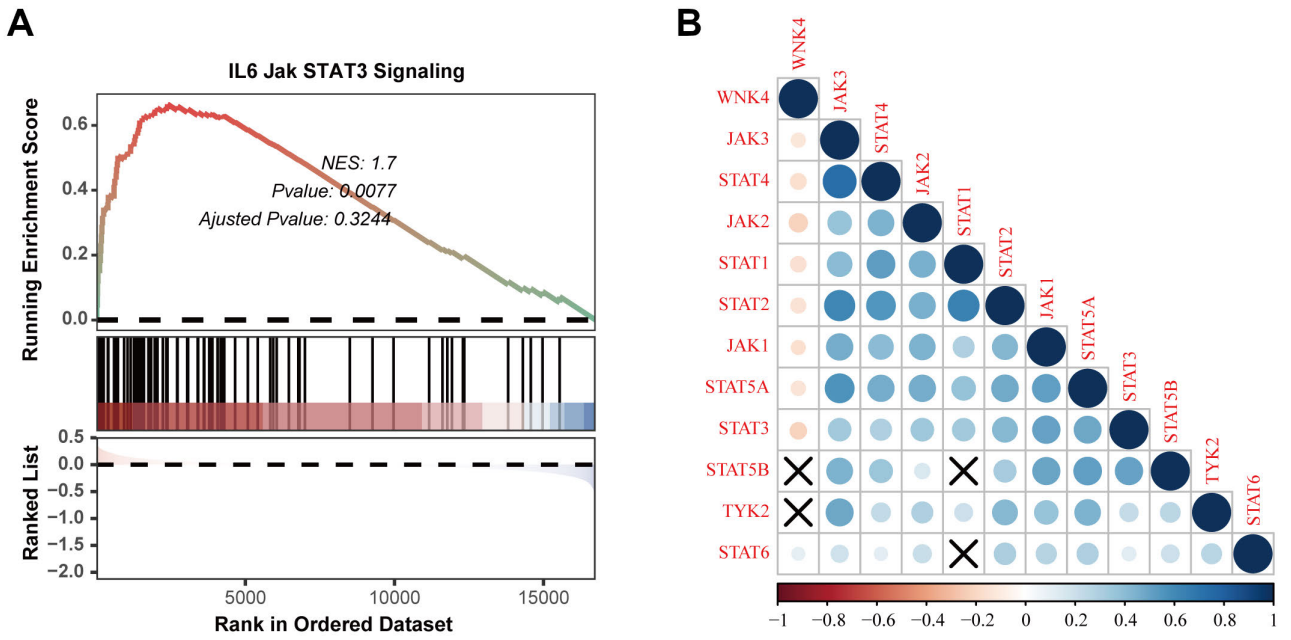



**Figure 4. The close association between WNK4 expression and the JAK-STAT3 signaling pathway. A) GSEA analysis of the GC gene expression profiles from TCGA database showed that low expression of WNK4 was positively correlated with the IL-6/JAK/STAT3 pathway gene set (NES=1.7, p<0.05). B) Heatmap of the correlation between WNK4 and key genes of the JAK-STAT pathway. Red represents a negative correlation; blue represents a positive correlation. The larger the correlation r value, the larger the circle. The x represents no significance.**

function [26]. Stattic used in this study was provided by Med Chem Express (MCE, #HY-13818), and the experimental concentration of Stattic was 10 μM. The results depicted in Figure 5C demonstrated that Stattic significantly attenuated STAT3 phosphorylation in GC cells exhibiting low WNK4 expression. CCK-8 assay and colony-forming experiment suggested that blockade of the STAT3 signaling pathway promoted cell death in WNK4 knockdown MKN45 cells (Figures 5D, 5E). These findings imply that the low expression of WNK4 activates the STAT3 signaling pathway and drives tumorigenesis in GC (Figure 5F).

## Discussion

The WNK family represents a novel class of serine/threonine protein kinases [27]. The name "WNK" was derived from the absence of an ATP-binding lysine residue on the second subunit of its kinase domain, which distinguished it from other kinases. In WNK kinases, the beta chain of lysine residues within the structural domain subunit 1 played a crucial role in mediating ATP binding and phosphorylation events. Adjacent to the kinase domain lies an autoinhibitory domain that maintains WNK kinase activity at a low level through autophosphorylation processes [28]. Increasing evidence suggested aberrant expression of WNK in tumor tissues, implicating its involvement in tumor growth, metastasis, and angiogenesis [29]. This article focused on elucidating the WNK4 in GC.

We observed for the first time that WNK4 protein and mRNA were expressed at lower levels in GC samples than those in normal tissues and cells, as determined using a large public database. To validate these findings, WB and RT-PCR were employed to determine WNK4 expression in GC cells and normal human gastric epithelial cells. The results demonstrated a substantial decrease in WNK4 expression within GC cells in contrast to GES-1, which corroborated data from TCGA and HPA databases. Moreover, analysis of TCGA database revealed a correlation between low WNK4 expression and advanced tumor stage, grade, as well as poor clinical outcome. AGS cells with relatively low endogenous WNK4 expression were selected for overexpression experiments. Our data showed that GC cells overexpressing WNK4 exhibited significantly reduced proliferative capacity. Conversely, MKN45 cells with relatively high endogenous WNK4 expression were chosen for knockdown experiments, resulting in enhanced proliferation ability of GC cells with suppressed levels of WNK4. It was noteworthy that different members within the WNK family played distinct roles across various types of tumors. Previous study has reported high expression levels of WNK1 in HCC, where it was related to poor prognosis [30]. Additionally, research on colon cancer cell lines has shown that silencing or inhibiting WNK1 effectively suppressed both *in vitro* and *in vivo* proliferation of colon cancer [31]. Downregulation of WNK1 expression in various breast cancer models has demonstrated a reduction in tumor migration, invasion, demonstrating that targeting WNK1

could serve as a strategy for mitigating the progression of invasive breast cancer [32]. Decreased expression of WNK2 has been correlated with tumor recurrence and adverse OS outcomes in patients with HCC [19]. Suppression of WNK2 in the glioblastoma SW1088 cell line enhanced cell migration and invasion and soft agar colony formation. Conversely, the reintroduction of WNK2 into A172H glioblastoma cells obstructed their capacity to establish clusters in soft agar and

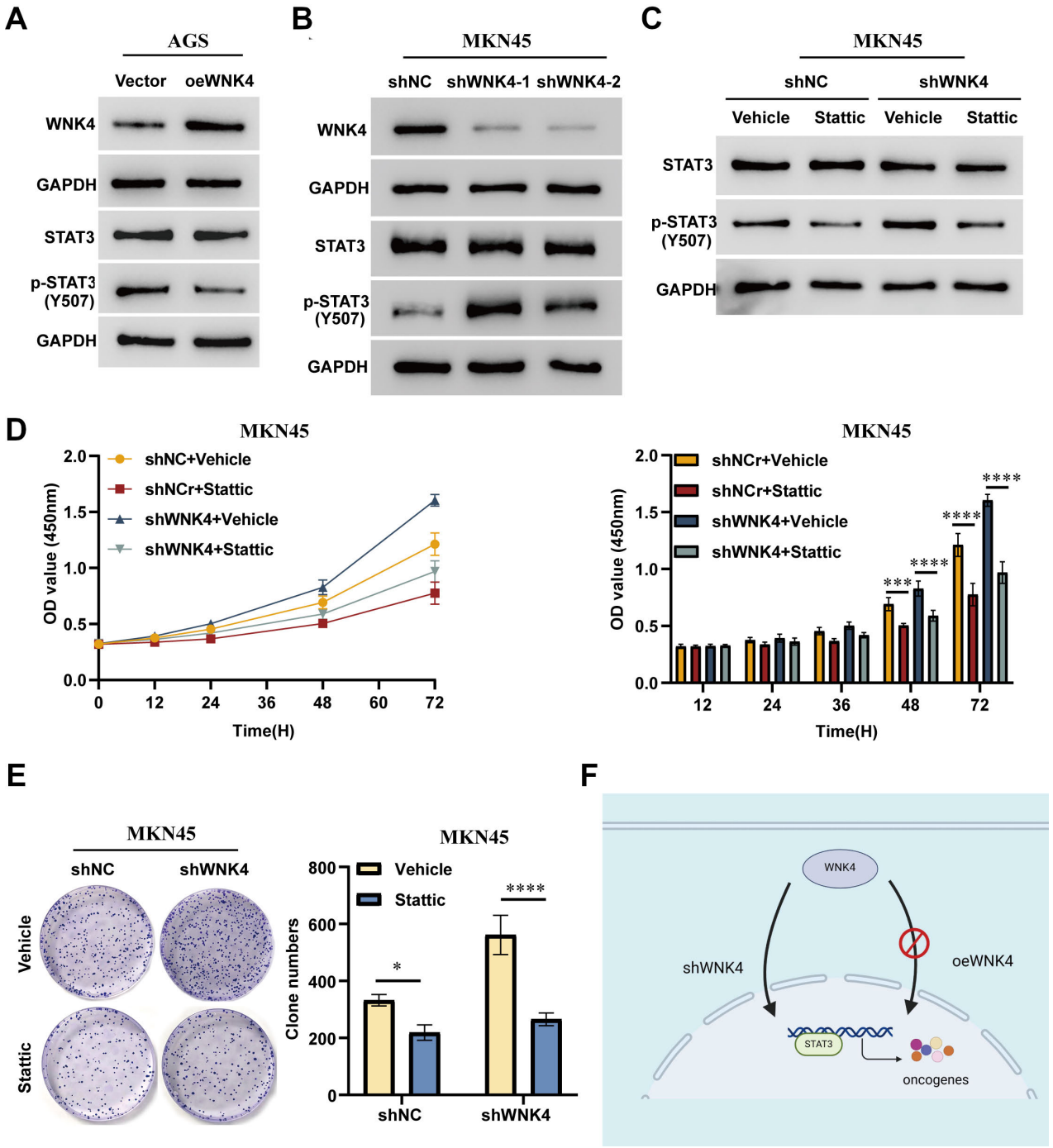



**Figure 5. Knockdown of WNK4 activated the STAT3 signaling pathway in GC cells. A) The protein levels of STAT3 and p-STAT3 (Y705) were assessed via WB analysis in AGS cells overexpressing WNK4. B) WNK4 knockdown-MKN45 cells were also examined for the aforementioned protein levels. C) The effect of Stattic treatment on WNK4 knockdown-MKN45 cells was evaluated by measuring the protein expression of STAT3 and p-STAT3 (Y705). Functional assays including CCK-8 assay D) and colony formation assay E) demonstrated that Stattic effectively reversed the growth and proliferative capacity induced by WNK4 knockdown in GC cells. F) Schematic representation of WNK4 influencing GC progression through STAT3. Data were expressed as the mean ± SD; *p<0.05, ***p<0.001, ****p<0.0001**

suppressed tumor development in a chick chorioallantoic tumor model [33]. This evidence backed WNK2 as a potent tumor suppressor. It can be seen as a dual function for WNK proteins in tumorigenesis. Interestingly, in both medulloblastoma and gastric cancer, STAT3 appears to be a key transcriptional regulator, while they differ in WNK4 expression. Upregulation of WNK4 enhanced medulloblastoma cell proliferation and confers resistance to cisplatin treatment, contributing to poor prognosis. However, as we found, upregulation of WNK4 inhibited the proliferation of GC cells.

Next, we investigate the mechanisms of regulating GC after WNK4 knockdown. GSEA revealed a negative correlation between WNK4 and the STAT3 signaling pathway. This was further validated through cell experiments, where in overexpression of WNK4 led to a notable reduction in p-STAT3 expression in GC cells, which was substantially increased when WNK4 was knocked down. Furthermore, we observed that STAT3 inhibitors could counteract the effects of WNK4 knockdown on GC cells. Here, we concluded that WNK4 suppression may induce GC proliferation through activation of the STAT3. Hyperactivation of the STAT3 pathway has been observed in various human tumors such as HCC and CRC, classifying it as an oncogene [34, 35]. RBMS1 promoted GC metastasis via autocrine IL-6/JAK2/STAT3 signaling [36], the acceleration of GC cell proliferation and invasion was facilitated by circBGN through the activation of the IL-6/STAT3 pathway [37], and Kras or BRAF mutation synergize with STAT3 activation contribute to GC progression [38]. Altogether, these highlighted a strong connection between the STAT3 pathway and GC.

We revealed a novel mechanism underlying gastric carcinogenesis. However, the specific mechanisms by which reduced expression of WNK4 induced activation of the STAT3 in GC remain unclear, and further investigations will be conducted to address this question.

Our findings not only provided deeper insights into the role of WNK4 in GC but also presented the first evidence suggesting that WNK4 may function as a tumor suppressor gene. Therefore, WNK4 could be considered a promising new target for optimizing clinical practice.

**Supplementary information** is available in the online version of the paper.

Acknowledgments: The research was funded by the National Natural Science Foundation of China (81973346) and the Jiangsu Province medical key discipline project (ZDXK202237).

## References

[1] SUNG H, FERLAY J, SSIEGEL RL, LAVERSANNE M, SOERJOMATARAM I et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 2021; 71: 209–249. https://doi.org/10.3322/caac.21660

[2] CATS A, JANSEN EPM, VAN GRIEKEN NCT, SIKORSKA K, LIND P et al. Chemotherapy versus chemoradiotherapy after surgery and preoperative chemotherapy for resectable gastric cancer (CRITICS): an international, open-label, randomised phase 3 trial. Lancet Oncol 2018; 19: 616–628. https://doi.org/10.1016/S1470-2045(18)30132-3

[3] SITARZ R, SKIERUCHA M, MIELKO J, OFFERHAUS GJA, MACIEJEWSKI R et al. Gastric cancer: epidemiology, prevention, classification, and treatment. Cancer Manag Res 2018; 10: 239. https://doi.org/10.2147/CMAR.S149619

[4] CAO Y, JIAO N, SUN T, MA Y, ZHANG X et al. CXCL11 Correlates With Antitumor Immunity and an Improved Prognosis in Colon Cancer. Front Cell Dev Biol 2021; 9: 646252. https://doi.org/10.3389/fcell.2021.646252

[5] KIM ST, CRISTESCU R, BASS AJ, KIM KM, ODEGAARD JI et al. Comprehensive molecular characterization of clinical responses to PD-1 inhibition in metastatic gastric cancer. Nat Med 2018; 24: 1449–1458. https://doi.org/10.1038/s41591-018-0101-z

[6] ZENG Y, JIN RU. Molecular pathogenesis, targeted therapies, and future perspectives for gastric cancer. Semin Cancer Biol 2022; 86: 566–582. https://doi.org/10.1016/j.semcancer.2021.12.004

[7] GAO Q, WOLFGANG MJ, NESCHEN S, MORINO K, HORVATH TL et al. Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. Proc Natl Acad Sci U S A 2004; 101: 4661–4666. https://doi.org/10.1073/pnas.0303992101

[8] INOUE H, OGAWA W, OZAKI M, HAGA S, MATSUMOTO M et al. Role of STAT-3 in regulation of hepatic gluconeogenic genes and carbohydrate metabolism in vivo. Nat Med 2004; 10: 168–174. https://doi.org/10.1038/nm980

[9] OLLILA S, DOMÈNECH-MORENO E, LAAJANEN K, WONG IP, TRIPATHI S et al. Stromal Lkb1 deficiency leads to gastrointestinal tumorigenesis involving the IL-11-JAK/STAT3 pathway. J Clin Invest 2018; 128: 402–414. https://doi.org/10.1172/JCI93597

[10] LI W, ZHANG X, WU F, ZHOU Y, BAO Z et al. Gastric cancer-derived mesenchymal stromal cells trigger M2 macrophage polarization that promotes metastasis and EMT in gastric cancer. Cell Death Dis 2019; 10: 918. https://doi.org/10.1038/s41419-019-2131-y

[11] SOUTTO M, CHEN Z, BHAT AA, WANG L, ZHU S et al. Activation of STAT3 signaling is mediated by TFF1 silencing in gastric neoplasia. Nat Commun 2019; 10: 3039. https://doi.org/10.1038/s41467-019-11011-4

[12] SUN TT, TANG JY, DU W, ZHAO HJ, ZHAO G et al. Bidirectional regulation between TMEFF2 and STAT3 may contribute to Helicobacter pylori-associated gastric carcinogenesis. Int J Cancer 2015; 136: 1053–1064. https://doi.org/10.1002/ijc.29061

[13] HOU G, ZUO H, SHI J, DAI D, WANG H et al. EIF4A3 induced circABCA5 promotes the gastric cancer progression by SPI1 mediated IL6/JAK2/STAT3 signaling. Am J Cancer Res 2023; 13: 602–622.

[14] COSTA AM, PINTO F, MARTINHO O, OLIVEIRA MJ, JORDAN P et al. Silencing of the tumor suppressor gene WNK2 is associated with upregulation of MMP2 and JNK in gliomas. Oncotarget 2015; 6: 1422–1434. https://doi.org/10.18632/oncotarget.2805

[15] XU B, ENGLISH JM, WILSBACHER JL, STIPPEC S, GOLDSMITH EJ et al. WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II. J Biol Chem 2000; 275: 16795–16801. https://doi.org/10.1074/jbc.275.22.16795

[16] VERÍSSIMO F, JORDAN P. WNK kinases, a novel protein kinase subfamily in multi-cellular organisms. Oncogene 2001; 20: 5562–5569. https://doi.org/10.1038/sj.onc.1204726

[17] JUN P, HONG C, LAL A, WONG JM, MCDERMOTT MW et al. Epigenetic silencing of the kinase tumor suppressor WNK2 is tumor-type and tumor-grade specific. Neuro Oncol 2009; 11: 414–422. https://doi.org/10.1215/15228517-2008-096

[18] SIE ZL, LI RY, SAMPURNA BP, HSU PJ, LIU SC et al. WNK1 Kinase Stimulates Angiogenesis to Promote Tumor Growth and Metastasis. Cancers (Basel) 2020; 12: 575. https://doi.org/10.3390/cancers12030575

[19] ZHOU SL, ZHOU ZJ, HU ZQ, SONG CL, LUO YJ et al. Genomic sequencing identifies WNK2 as a driver in hepatocellular carcinoma and a risk factor for early recurrence. J Hepatol 2019; 71: 1152–1163. https://doi.org/10.1016/j.jhep.2019.07.014

[20] WANG Y, WU B, LONG S, LIU Q, LI G. WNK3 promotes the invasiveness of glioma cell lines under hypoxia by inducing the epithelial-to-mesenchymal transition. Transl Neurosci 2021; 12: 320–329. https://doi.org/10.1515/tnsci-2020-0180

[21] GUERREIRO AS, FATTET S, KULESZA DW, ATAMER A, ELSING AN et al. A sensitized RNA interference screen identifies a novel role for the PI3K p110γ isoform in medulloblastoma cell proliferation and chemoresistance. Mol Cancer Res 2011; 9: 925–935. https://doi.org/10.1158/1541-7786.MCR-10-0200

[22] DENSON LA. Adding fuel to the fire: STAT3 priming of gastric tumorigenesis. Gastroenterology 2006; 131: 1342–1344. https://doi.org/10.1053/j.gastro.2006.08.049

[23] JUDD LM, BREDIN K, KALANTZIS A, JENKINS BJ, ERNST M et al. STAT3 activation regulates growth, inflammation, and vascularization in a mouse model of gastric tumorigenesis. Gastroenterology 2006; 131: 1073–1085. https://doi.org/10.1053/j.gastro.2006.07.018.

[24] MA Z, SUN Q, ZHANG C, ZHENG Q, LIU Y et al. RHOJ Induces Epithelial-to-Mesenchymal Transition by IL-6/STAT3 to Promote Invasion and Metastasis in Gastric Cancer. Int J Biol Sci 2023; 19: 4411–4426. https://doi.org/10.7150/ijbs.81972

[25] THILAKASIRI P, O'KEEFE RN, TO SQ, CHISANGA D, EISSMANN MF et al. Mechanisms of cellular crosstalk in the gastric tumor microenvironment are mediated by YAP1 and STAT3. Life Sci Alliance 2024; 7: e202302411. https://doi.org/10.26508/lsa.202302411

[26] SCHUST J, SPERL B, HOLLIS A, MAYER TU, BERG T. Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. Chem Biol 2006; 13: 1235–1242. https://doi.org/10.1016/j.chembiol.2006.09.018

[27] BOYD-SHIWARSKI CR, SHIWARSKI DJ, GRIFFITHS SE, BEACHAM RT, NORRELL L et al. WNK kinases sense molecular crowding and rescue cell volume via phase separation. Cell 2022; 185: 4488–4506.e20. https://doi.org/10.1016/j.cell.2022.09.042

[28] RODAN AR, JENNY A. WNK Kinases in Development and Disease. Curr Top Dev Biol 2017; 123 :1–47. https://doi.org/10.1016/bs.ctdb.2016.08.004

[29] MONIZ S, JORDAN P. Emerging roles for WNK kinases in cancer. Cell Mol Life Sci 2010; 67: 1265–1276. https://doi.org/10.1007/s00018-010-0261-6

[30] WANG F, YAN X, SHI G, ZHANG L, JING X. Loss of WNK1 Suppressed the Malignant Behaviors of Hepatocellular Carcinoma Cells by Promoting Autophagy and Activating AMPK Pathway. Dis Markers 2022; 2022: 6831224. https://doi.org/10.1155/2022/6831224

[31] JAYKUMAR AB, JUNG JU, PARIDA PK, DANG TT, WICHAIDIT C et al. WNK1 Enhances Migration and Invasion in Breast Cancer Models. Mol Cancer Ther 2021; 20: 1800–1808. https://doi.org/10.1158/1535-7163.MCT-21-0174

[32] JIANG H, CHENG X, LIANG Y, WANG Y, LI Y et al. Aberrant expression of WNK lysine deficient protein kinase 1 is associated with poor prognosis of colon adenocarcinoma. Ir J Med Sci 2023; 192: 57–64. https://doi.org/10.1007/s11845-021-02916-5

[33] MONIZ S, MARTINHO O, PINTO F, SOUSA B, LOUREIRO C et al. Loss of WNK2 expression by promoter gene methylation occurs in adult gliomas and triggers Rac1-mediated tumour cell invasiveness. Hum Mol Genet 2013; 22: 84–95. https://doi.org/10.1093/hmg/dds405

[34] FENG DY, ZHENG H, TAN Y, CHENG RX. Effect of phosphorylation of MAPK and Stat3 and expression of c-fos and c-jun proteins on hepatocarcinogenesis and their clinical significance. World J Gastroenterol 2001; 7: 33–36. https://doi.org/10.3748/wjg.v7.i1.33

[35] MA XT, WANG S, YE YJ, DU RY, CUI ZR et al. Constitutive activation of Stat3 signaling pathway in human colorectal carcinoma. World J Gastroenterol 2004; 10: 1569–1573. https://doi.org/10.3748/wjg.v10.i11.1569

[36] LIU M, LI H, ZHANG H, ZHOU H, JIAO T et al. RBMS1 promotes gastric cancer metastasis through autocrine IL-6/JAK2/STAT3 signaling. Cell Death Dis 2022; 13: 287. https://doi.org/10.1038/s41419-022-04747-3

[37] LI C, PENG X, PENG Z, YAN B. circBGN accelerates gastric cancer cell proliferation and invasion via activating IL6/STAT3 signaling pathway. FASEB J 2022; 36: e22604. https://doi.org/10.1096/fj.202200957RR

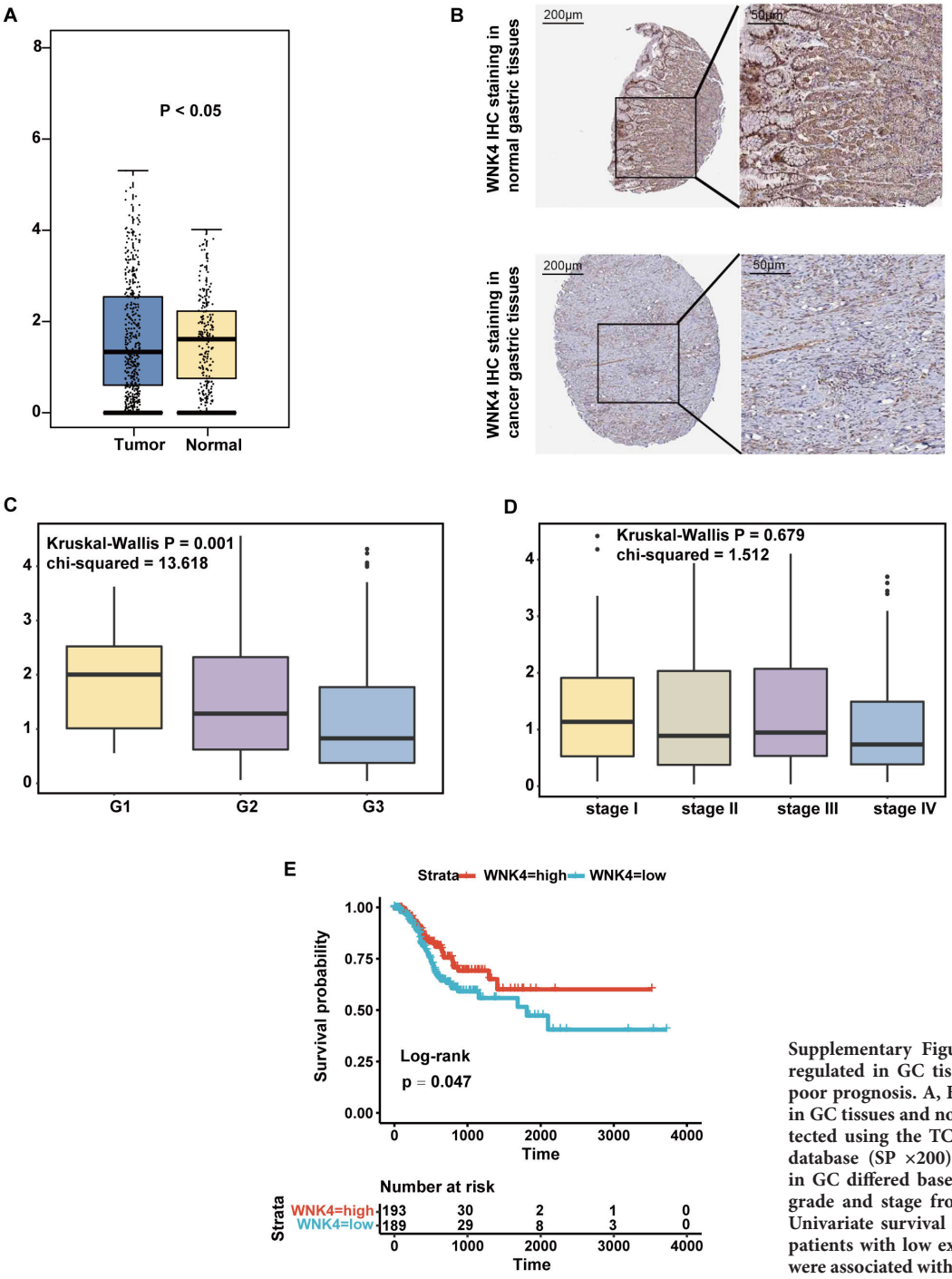
[38] THIEM S, EISSMANN MF, ELZER J, JONAS A, PUTOCZKI TL et al. Stomach-Specific Activation of Oncogenic KRAS and STAT3-Dependent Inflammation Cooperatively Promote Gastric Tumorigenesis in a Preclinical Model. Cancer Res 2016; 76: 2277–2287. https://doi.org/10.1158/0008-5472.CAN-15-3089

https://doi.org/10.4149/neo_2024_240220N67

# Downregulation of WNK4 expression facilitates the proliferation of gastric cancer cells via activation of the STAT3 signaling pathway

Miao LI[1,#], Xiaoyan SHAO[2,#], Qiqi NING[3], Rongrong SUN[2], Rantian LI[2], Yanhua LIU[2], Yuan YUAN[2,*], Youwei ZHANG[1,2,*]

## Supplementary Information




**Supplementary Figure S1. WNK4 was downregulated in GC tissues and associated with a poor prognosis. A, B) The expression of WNK4 in GC tissues and normal gastric tissues was detected using the TCGA database and the HPA database (SP ×200). C, D) WNK4 expression in GC differed based on the individual cancer grade and stage from the TCGA database. E) Univariate survival analysis demonstrated that patients with low expression of WNK4 protein were associated with adverse OS.**

**Supplementary Table S1.** shRNAs sequences for Lentivirus infection.

| | **Sequences** |
|---|---|
| WNK4-1 | SS Seq: 5'-GCTGCTGAAGACACCCTAA-3' |
| | AS Seq: 5'-TTAGGGTGTCTTCAGCAGC-3' |
| WNK4-1F | 5'-CCGGTGCTGCTGAAGACACCCTAACTCGAGTTAGGGTGTCTTCAGCAGCTTTTTG-3' |
| WNK4-1R | 5'-AATTCAAAAAGCTGCTGAAGACACCCTAACTCGAGTTAGGGTGTCTTCAGCAGCA-3 |
| WNK4-2 | SS Seq: 5'-GGTGAAGGAGATCATTGAA-3' |
| | AS Seq: 5'-TTCAATGATCTCCTTCACC-3' |
| WNK4-2F | 5'-CCGGTGGTGAAGGAGATCATTGAACTCGAGTTCAATGATCTCCTTCACCTTTTTG-3' |
| WNK4-2R | 5'-AATTCAAAAAGGTGAAGGAGATCATTGAACTCGAGTTCAATGATCTCCTTCACCA-3 |
| WNK4-3 | SS Seq: 5'-GGATCTCAAGTGCGACAAT-3' |
| | AS Seq: 5'-ATTGTCGCACTTGAGATCC-3' |
| WNK4-3F | 5'-CCGGTGGATCTCAAGTGCGACAATCTCGAGATTGTCGCACTTGAGATCCTTTTTG-3' |
| WNK4-3R | 5'-AATTCAAAAAGGATCTCAAGTGCGACAATCTCGAGATTGTCGCACTTGAGATCCA-3 |