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EFNA4 deletion suppresses the migration, invasion, stemness, and angiogenesis of gastric cancer cells through the inactivation of Pygo2/Wnt signaling

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Summary. Gastric cancer represents an aggressive malignancy and a leading contributor to cancer death. Ephrin-A4 (EFNA4) has been proposed to be related to the immune microenvironment and prognosis of gastric cancer. This study was undertaken to discuss the participation and mechanism of EFNA4 in the development of gastric cancer. RT-qPCR and western blot examined EFNA4 and Pygopus2 (Pygo2) expression in gastric cancer cells. After transfection of EFNA4 interference plasmids or co-transfection of EFNA4 interference plasmids and Pygo2 overexpression plasmids, cell proliferation was detected by the CCK-8 method and EDU staining. Wound healing, Transwell, TUNEL, and endothelial cell tube formation assays detected cell migration, invasion, apoptosis, and angiogenesis, respectively. Western blot examined the expression of metastasis-, apoptosis-, angiogenesis-, and Wnt signaling-associated proteins. Cell stemness was estimated by the sphere formation assay, RT-qPCR, and western blot. Through the experimental data, it was noticed that EFNA4 expression was increased in gastric cancer cells. Knockdown of EFNA4 suppressed the proliferation, migration, invasion, angiogenesis as well as stemness while aggravating the apoptosis of gastric cancer cells. Also, EFNA4 depletion reduced Pygo2 protein expression and then inactivated Wnt/β-catenin signaling. Further elevation of Pygo2 reversed the impacts of EFNA4 silencing on Wnt/β-catenin signaling, cell proliferation, apoptosis, migration, invasion, angiogenesis as well as stemness in gastric cancer. Accordingly, the knockdown of EFNA4 might downregulate Pygo2 and inactivate Wnt/β-catenin signaling to exert protective effects against gastric cancer.

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Introduction

Gastric cancer refers to a highly prevalent aggressive tumor of the gastrointestinal system (Yang et al., 2021). As reported by global cancer statistics from 2023, more than 26,500 people were diagnosed with gastric cancer and approximately 11,130 deaths resulted from gastric cancer annually worldwide (Siegel et al., 2023). Early gastric cancer is a curable disease through surgery. Unfortunately, gastric cancer patients at advanced stages account for the majority attributed to the atypical early symptoms, leading to a pessimistic five-year survival rate (Sitarz et al., 2018). Also, some patients may not respond or may develop resistance to chemotherapy or radiotherapy (Zhang et al., 2022). Moreover, tumor relapse remains a major driver of therapeutic failure and poor survival in gastric cancer (Jiao et al., 2020). Considering that the optimal choice of regimen is still uncertain, there is a strong demand for a better understanding of the molecular events critical to gastric cancer progression and adopting therapeutic measures to improve the prognosis and treatment of gastric cancer.

Ephrins are membrane-bound proteins implicated in diversified physiological and pathological events, such as neuronal plasticity, homeostatic events, and disease processes (Arcas et al., 2020; Bush, 2022). Ephrin-A4 (EFNA4), belonging to the Ephrin family, extensively exists in a wide range of human organs including spleen, lymph nodes, ovary, small intestine, and colon (Lin et al., 2021). Recently, accumulative evidence has identified EFNA4 as an oncogene in hepatocellular carcinoma (Lin et al., 2021), oral squamous cell carcinoma (Chen et al., 2021), and glioma (Miao et al., 2015). Previous literature has supported that EFNA4 expression is upregulated in gastric cancer tissues compared with normal adjacent tissues and is related to overall survival and disease-free survival and tumor-

infiltrating immune cell levels in patients with gastric cancer (Ji et al., 2021; Xie et al., 2022). Based on the GSE103236 dataset from the GEO database, EFNA4 expression is also increased in stomach adenocarcinoma (STAD) tissues (Yu et al., 2020; Yang and Gong, 2021). However, studies regarding the role of EFNA4 in the course of gastric cancer remain scarce.

As a member of the Pygopus family of proteins, a novel element of the Wingless (Wg) pathway in *Drosophila*, mammalian Pygopus2 (Pygo2) has been found to possess aberrantly elevated expression in human malignancies, such as breast cancer, kidney cancer, esophageal squamous cell carcinoma, etc., and is responsible for aggressive cellular behaviors, such as cell growth, metastasis, invasion, etc. (Liu et al., 2015; Chi et al., 2019; Ardalan Moghadam Al et al., 2024). Additionally, Pygo2 may be a promising biomarker to monitor drug resistance in gastric cancer (Zhang et al., 2021). Pygo2 can be downregulated by ibuprofen to inactivate the Wnt signaling pathway and suppress cell proliferation in gastric cancer (Akrami et al., 2018). It is well documented that Pygo2 is a novel functional protein downstream of the Wnt signaling pathway (Andrews and Kao, 2016; Talla and Brembeck, 2016). Moreover, Yuan et al. reported that EFNA4 silencing can downregulate Pygo2 to suppress the malignant process of hepatocellular carcinoma (Yuan et al., 2022).

The present report is to explore the involvement of EFNA4 in gastric cancer related to Pygo2/Wnt signaling.

Materials and methods

Cell culture and treatment

The human gastric epithelium GES-1 cell line, gastric cancer cell lines (KE-39, MKN45, and AGS), and human umbilical vein endothelial cells (HUVECs) were all procured from CoBioer Biosciences Co., Ltd. (Nanjing, China). The GES-1 cell line was incubated in Dulbecco's modified Eagle medium (DMEM; Basal Media Biotechnology, Shanghai, China), while all gastric cancer cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Basal Media Biotechnology, Shanghai, China). All cells were cultivated with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biotechnology, Hangzhou, China) in a 5% CO_2-saturated humid environment at 37°C, except that the medium of MKN45 cells was supplemented with 20% FBS for incubation. The endothelial cell medium (ECM; ScienCell, USA) containing growth supplements was prepared for HUVEC cultivation.

Transfection of plasmids

Specific small interfering RNAs (siRNAs) for EFNA4 (si-EFNA4#1/2; Bioneer, Daejeon, South Korea), which were ligated into the pi- GENE™hU6 Puro plasmid vector (Clontech, USA), and Pygo2 overexpression plasmids (OE-Pygo2), by ligating Pygo2 cDNA into pcDNA3.1 plasmids (VectorBuilder, Inc.), as well as their respective negative control si-NC and OE-NC, were transfected into AGS cells with Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. After lentivirus infection, AGS cells were treated with 1 mg/ml puromycin to obtain stably transfected cell lines. Cells were cultured for 24h before transfection. Cells were transfected when cells reached 70-80% confluency. After 48h, cells were collected.

Cell Counting Kit-8 (CCK-8)

Before the addition of 10 µl CCK-8 solution (Selleck Chemicals, US) at designated time-points (24, 48, and 72h) for 2h of cultivation, the density of transfected AGS cells placed into 96-well plates was adjusted to 5×10^3 cells/well. Cell survival was assayed by reading the OD 450 nm value with a microplate reader (Diatek, Wuxi, China).

5-Ethynyl-2'-deoxyuridine (EdU) staining

Cell proliferation was measured via the Cell-Light™ EdU Cell Proliferation Detection Assay (LifeSpan BioSciences, Inc.). In brief, the transfected AGS cells (5×10^4 cells/well) were inoculated in 96-well plates for 24h and labeled with 20 µM EDU for 2h, following the standard protocol. Afterward, cells were fixed in 4% paraformaldehyde for 15 min and permeated with 0.5% Triton X-100 for 20 min and then probed with iClick EdU reaction buffer for 30 min and stained with Hoechst 33342 for 15 min. Under a fluorescence microscope (Carl Zeiss, Germany), the EdU-positive cells were imaged.

Wound healing assay

AGS cells were cultured in 6-well plates (7×10^5 cells/well) for 24h. Upon cells reaching 90% confluency, the cell monolayer was wounded with a 200 µL micropipette tip. Cellular debris was removed after being washed with the fresh RPMI-1640 medium. The spread of wound closure at 0, 6, 12, and 24h was documented under an inverted microscope (Olympus, Tokyo, Japan).

Transwell assay

The suspension of AGS cells (200 µl) was loaded into the upper chambers of a polycarbonate Transwell filter (Cell Biolabs, Inc. Santiago, USA) coated with Matrigel (BD Biosciences, MA, USA) for 30 min. The undersides were filled with 500 µL medium with 10% FBS. After 24h, the remaining cells in the upper chamber were cleared while the cells that migrated or invaded to the bottom of the membrane were immobilized with methanol for 30 min and stained with 0.1% crystal violet for 10 min, and then counted under a light microscope.

Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling (TUNEL)

The DeadEnd™ Colorimetric TUNEL System Kit (Promega, Madison, WI) was employed to evaluate AGS cell apoptosis as per the manufacturer's instructions. After being immobilized with 4% paraformaldehyde for 20 min and permeated with 0.1% Triton X-100 for 10 min, AGS cells were incubated with the TUNEL reaction mixture for 1h shielded from light. Next, 10 mg/ml DAPI was added for nuclear staining for 10 min. Finally, the images were acquired under a fluorescence microscope.

Endothelial cell tube formation assay

After the conditioned medium was prepared by mixing the supernatants of AGS cells with fresh RPMI-1640 medium, HUVECs (3×10^4) were inoculated in the 96-well plates coated with Matrigel for 30 min in the conditioned medium. After 2 days, tubules were observed under an inverted microscope.

Sphere formation assay

The transfected AGS cells (5×10^3 cells per well) loaded into ultra-low attachment 24-well plates (Corning Incorporated, Corning, NY, USA) were maintained in serum-deprived RPMI-1640 medium supplemented with 20 ng/ml epidermal growth factor and 10 ng/ml basic fibroblast growth factor. Cells were maintained for 7 days at 37°C in a 5% CO_2 incubator. The tumor spheres were imaged under a light microscope and only spheres with a diameter over 50 μm were counted using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was purified from gastric cancer cells using Trizol reagent (AccuRef Scientific, Xi'an, China). Then cDNA was prepared with the Accuref 1st Strand cDNA Synthesis Kit (AccuRef Scientific, Xi'an, China) and subjected to PCR amplification with Accuref qPCR SYBR Green Mixture (AccuRef Scientific, Xi'an, China), relative gene expression was calibrated with the $2^{-\Delta\Delta Cq}$ approach. GAPDH functioned as a normalization gene.

Western blot

RIPA buffer (AccuRef Scientific, Xi'an, China) containing a 1% protease inhibitor cocktail (EMD Millipore) was used to extract the total protein from gastric cancer cells, which was prepared for concentration measurement using the BCA method (AccuRef Scientific, Xi'an, China). After separation on 12% SDS-PAGE, the proteins were transferred to PVDF membranes, which were then blocked with 5% BSA for 2h. Next, membranes were incubated with primary antibodies recognizing EFNA4 (cat. no. #AF0530, 1/1000; Affinity Biosciences), matrix metalloproteinase-2 (MMP2; cat. no. #AF5330, 1/1000; Affinity Biosciences), matrix metalloproteinase-9 (MMP9; cat. no. #AF5228, 1/1000; Affinity Biosciences), B-cell lymphoma 2 (BCL2; cat. no. #AF0769, 1/1000; Affinity Biosciences), Bcl-2-associated X (Bax; cat. no. #AF0120, 1/1000; Affinity Biosciences), Cleaved caspase-3 (cat. no. #AF7022, 1/1000; Affinity Biosciences), vascular endothelial growth factor (VEGF; cat. no. #AF5131, 1/1000; Affinity Biosciences), vascular endothelial growth factor receptor 2 (VEGFR2; cat. no. #AF6281, 1/1000; Affinity Biosciences), SRY-box 2 (Sox2; cat. no. #AF5140, 1/1000; Affinity Biosciences), octamer-binding transcription factor 4 (Oct4; cat. no. ab109183, 1/1000; Abcam), Nanog (cat. no. #AF5388, 1/1000; Affinity Biosciences), Pygo2 (cat. no. #DF8107, 1/1000; Affinity Biosciences), C-myc (cat. no. #AF6054, 1/1000; Affinity Biosciences), Cyclin D1 (cat. no. #AF0931, 1/1000; Affinity Biosciences), β-catenin (cat. no. #AF6266, 1/1000; Affinity Biosciences), and β-actin (cat. no. #AF7018; 1/3000; Affinity Biosciences) at 4°C overnight, as well as the corresponding secondary antibody horseradish peroxidase (HRP)-conjugated goat anti-rabbit Immunoglobulin G (#S0001; 1/3000; Affinity Biosciences) for 1h at room temperature. The immunoreactive products were developed by the super ECL kit (AccuRef Scientific, Xi'an, China). ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA) was used for densitometry analysis.

Statistical analyses

All experimental values, analyzed with GraphPad Prism 8.0 software (San Diego, CA, USA), were manifested as the mean ± SD. Difference comparisons among groups were performed by One-way ANOVA as well as Tukey's post-hoc test. $p<0.05$, $p<0.01$, $p<0.001$ denoted statistical significance.

Results

EFNA4 exhibits elevated expression in gastric cancer cells

To uncover the regulatory role of EFNA4 in gastric cancer, EFNA4 expression was analyzed by RT-qPCR and western blot. We found that EFNA4 mRNA and protein expression were both evidently increased in gastric cancer cell lines (KE-39, MKN45, and AGS) relative to the human gastric epithelium immortalized cell line GES-1 (Fig. 1A). AGS cells that displayed the highest EFNA4 expression were selected for the ensuing experiments. After transfection of EFNA4 knockdown plasmids (si-EFNA4#1/2), EFNA4 expression at both mRNA and protein levels was noted to be distinctly

depleted in AGS cells. Moreover, si-EFNA4#2 was chosen for the follow-up assays for its prominent interference efficacy (Fig. 1C).

Deletion of EFNA4 inhibits the proliferation, migration, and invasion of AGS cells

Using the CCK-8 assay, AGS cell viability was evaluated, observing that cell viability was markedly reduced after EFNA4 was silenced (Fig. 2A). The experimental data from EdU staining assay also showed that when EFNA4 was depleted, the number of EdU-positive AGS cells was noticeably reduced (Fig. 2B), suggesting that EFNA4 deficiency suppressed gastric cancer cell proliferation. Through the wound healing assay, it was noted that EFNA4 knockdown prominently lowered the migration rate of AGS cells (Fig. 2C). As Fig. 2D depicted, the results of the Transwell assay revealed that the invasive capacity of AGS cells was significantly weakened by EFNA4 silencing. Additionally, the expression of metastasis-associated proteins, including MMP2 and MMP9, were both markedly depleted upon knockdown of EFNA4 (Fig. 2E).

Deletion of EFNA4 promotes apoptosis and represses angiogenesis of AGS cells

Concurrently, the impacts of EFNA4 on AGS cell apoptosis were detected by the TUNEL staining assay. As portrayed in Figure 3A, the absence of EFNA4 apparently enhanced the number of TUNEL-positive cells, which implied that EFNA4 downregulation aggravated gastric cancer cell apoptosis. Western blot analysis also suggested that EFNA4 interference elevated Bax, Cleaved caspase-3/Caspase-3 expression, and reduced Bcl-2 expression in AGS cells (Fig. 3B). Furthermore, the endothelial cell tube formation assay and western blot analysis showed that EFNA4 depletion reduced the number of tubes and the expression of angiogenesis-related proteins, including VEGF and VEGFR2 (Fig. 3C,D), which hinted that EFNA4 inhibition exerted anti-angiogenetic activity on gastric cancer cells.

Deletion of EFNA4 suppresses the stemness of AGS cells

In addition, through the sphere formation assay, inhibition of EFNA4 notably decreased the sphere formation ability of cells (Fig. 4A), implying that silencing of EFNA4 played an inhibitory role in gastric cancer cell stemness. RT-qPCR and western blot further analyzed that mRNA and protein expression of stemness markers including Nanog, Sox2, and Oct4, were all decreased with EFNA4 inhibition (Fig. 4B-D).

Deletion of EFNA4 inactivates Pygo2/Wnt/β-catenin signaling

As reported, EFNA4 may participate in the process of hepatocellular carcinoma through upregulating Pygo2, which can mediate downstream Wnt signaling. As expected, western blot analysis also manifested that, after EFNA4 was depleted, Pygo2 protein expression also declined in AGS cells (Fig. 5A). Further

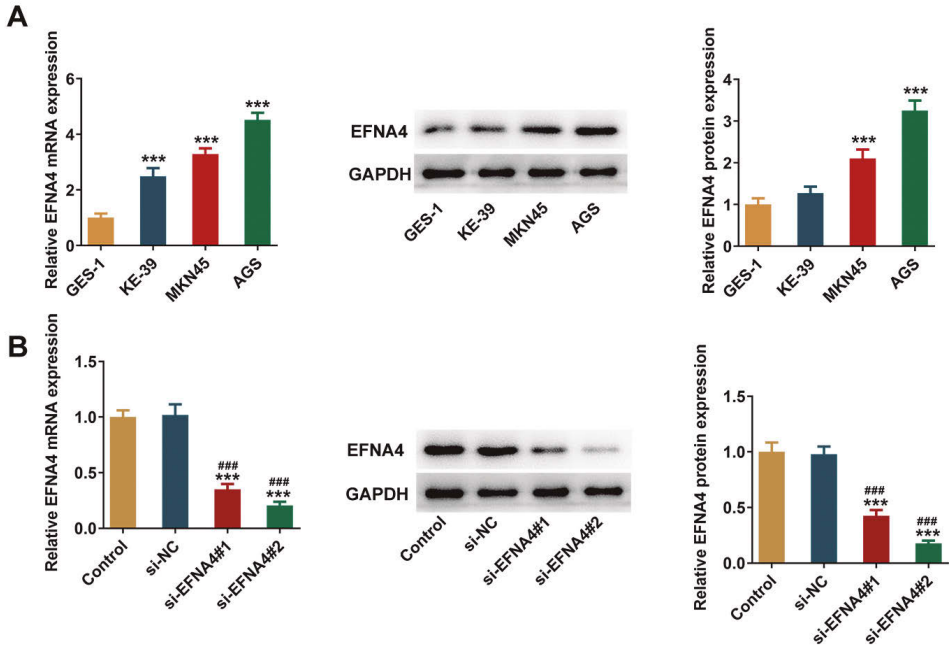

Fig. 1. EFNA4 exhibits elevated expression in gastric cancer cells. **A.** RT-qPCR and western blot analysis of EFNA4 expression in gastric cancer cells. ***P<0.001 vs. GES-1. **B.** Transfection efficacy of EFNA4 interference plasmids by RT-qPCR and western blot. ***p<0.001 vs. Control. ###p<0.001 vs. si-NC.

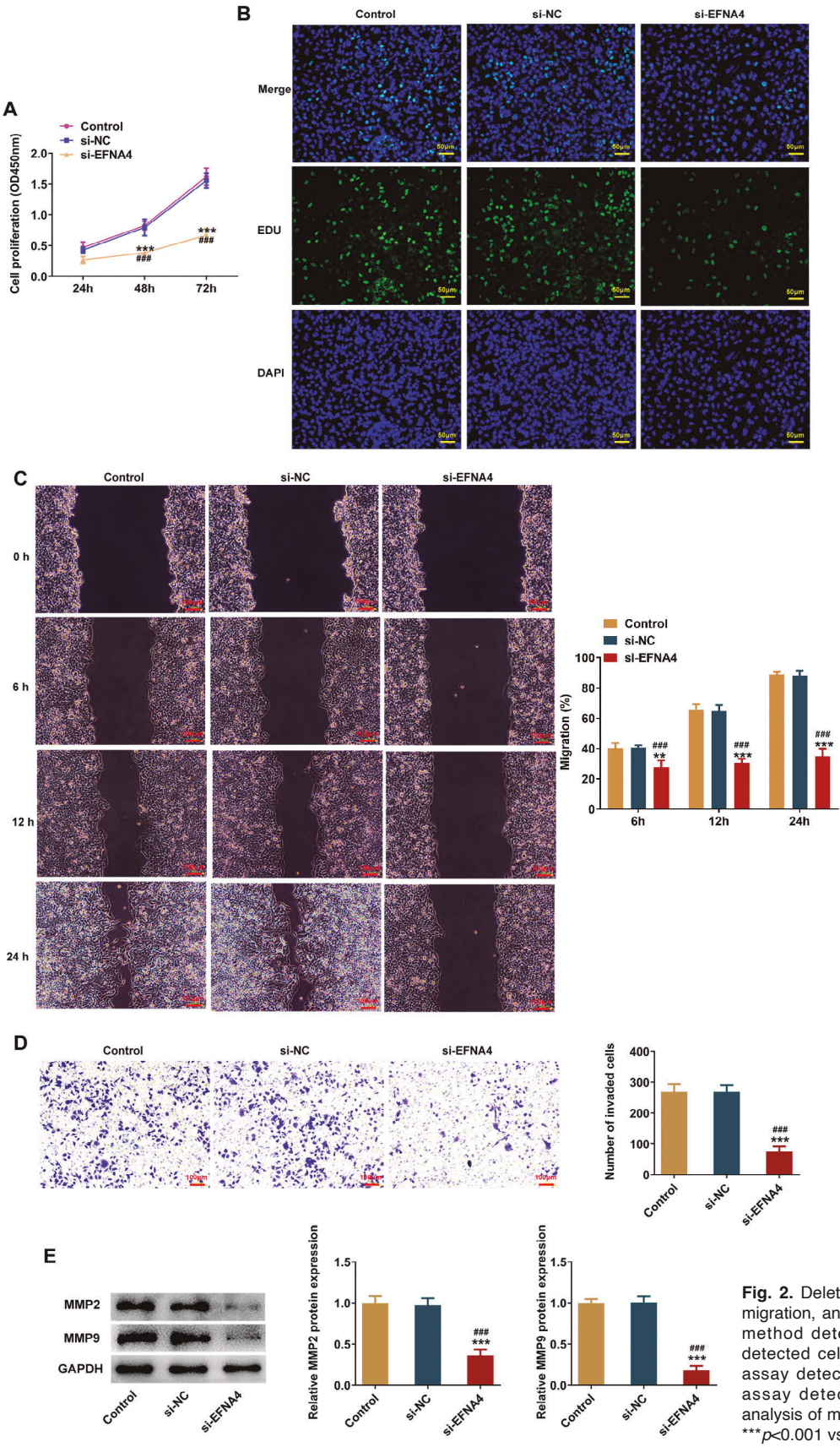

Fig. 2. Deletion of EFNA4 inhibits the proliferation, migration, and invasion of AGS cells. **A.** The CCK-8 method detected cell viability. **B.** EdU staining detected cell proliferation. **C.** The wound healing assay detected cell migration. **D.** The Transwell assay detected cell invasion. **E.** Western blot analysis of metastasis-associated proteins. $^{**}p<0.01$, $^{***}p<0.001$ vs. Control. $^{###}p<0.001$ vs. si-NC.

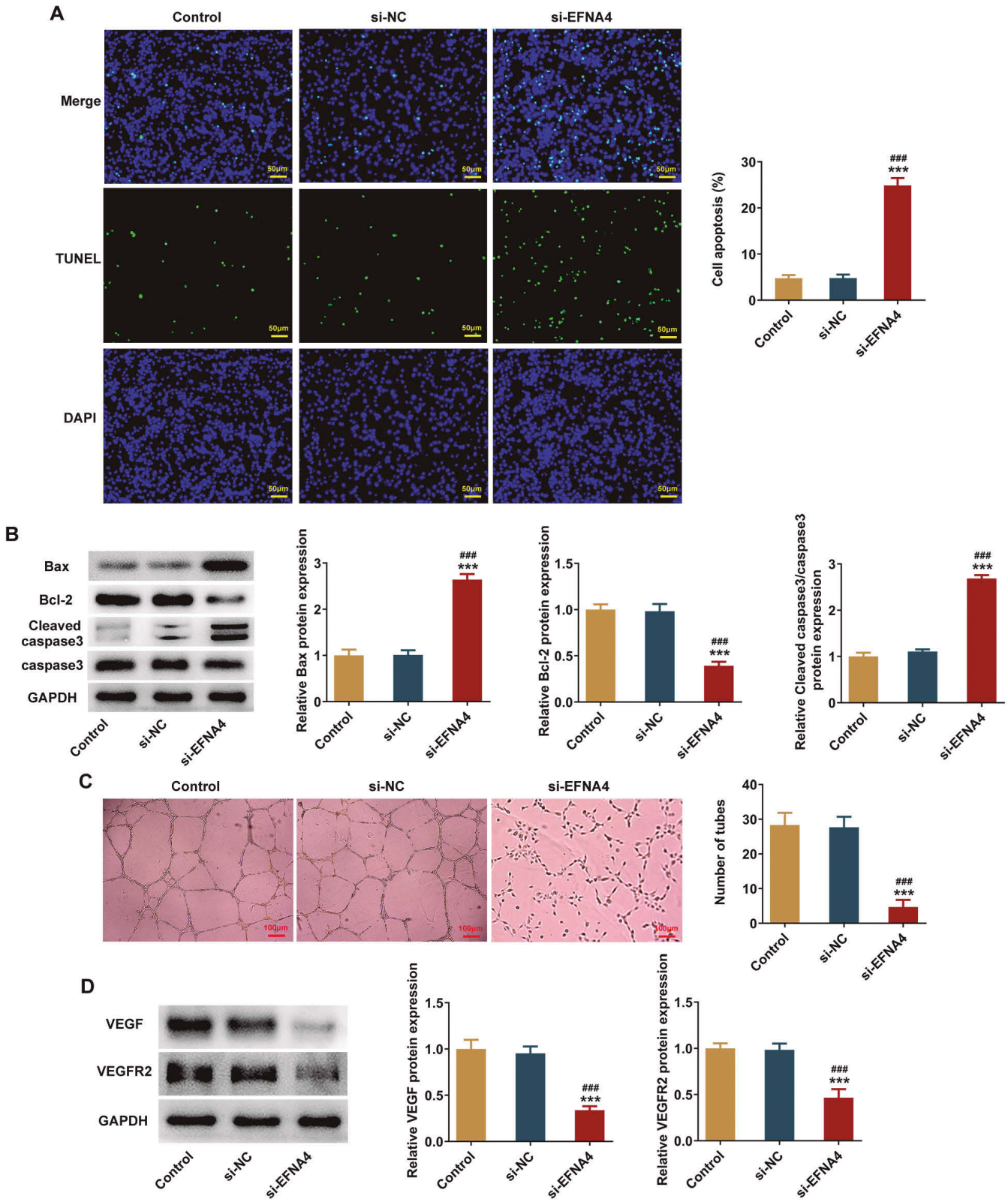

Fig. 3. Deletion of EFNA4 promotes apoptosis and represses the angiogenesis of AGS cells. **A.** TUNEL staining detected cell apoptosis. **B.** Western blot analysis of apoptosis-associated proteins. **C.** The endothelial cell tube formation assay detected cell angiogenesis. **D.** Western blot analysis of angiogenesis-associated proteins. $^{***}p<0.001$ vs. Control. $^{###}p<0.001$ vs. si-NC.

investigation clarified that EFNA4 deficiency clearly reduced the expression of Wnt/β-catenin signaling-associated proteins, including C-myc, Cyclin D1, and β-catenin (Fig. 5B), highlighting that EFNA4 might serve as an activator of Wnt/β-catenin signaling. After Pygo2 was overexpressed by transfection of OE-Pygo2 (Fig. 5C), the decreased C-myc, Cyclin D1, and β-catenin expression imposed by the absence of EFNA4 were all enhanced again (Fig. 5D).

EFNA4 deficiency downregulates Pygo2 to suppress the proliferation, migration, and invasion of AGS cells

Meanwhile, the results from CCK-8 and EDU staining assays manifested that the inhibited viability and proliferation of AGS cells on account of EFNA4

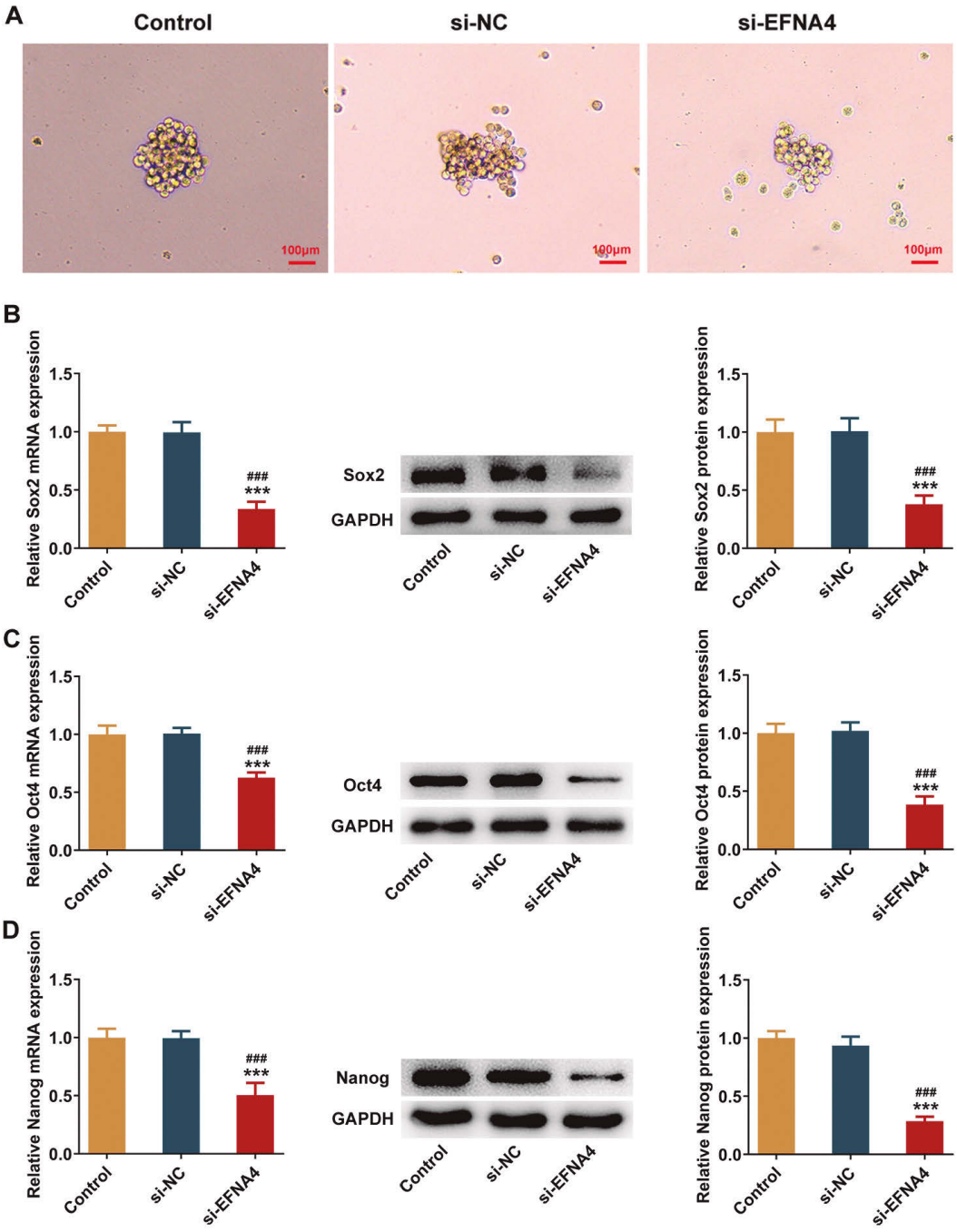

Fig. 4. Deletion of EFNA4 suppresses the stemness of AGS cells. **A.** The sphere formation assay measured cell stemness. **B.** RT-qPCR and western blot analysis of Sox2 expression. **C.** RT-qPCR and western blot analysis of Oct4 expression. **D.** RT-qPCR and western blot analysis of Nanog expression. ***$p<0.001$ vs. Control. ###$p<0.001$ vs. si-NC.

inhibition were both accelerated again when Pygo2 was upregulated (Fig. 6A,B). Also, the deletion of EFNA4 attenuated AGS cell migration and invasion abilities as well as downregulated MMP2 and MMP9 protein expression, which were all promoted by the elevation of Pygo2 (Fig. 6C-E).

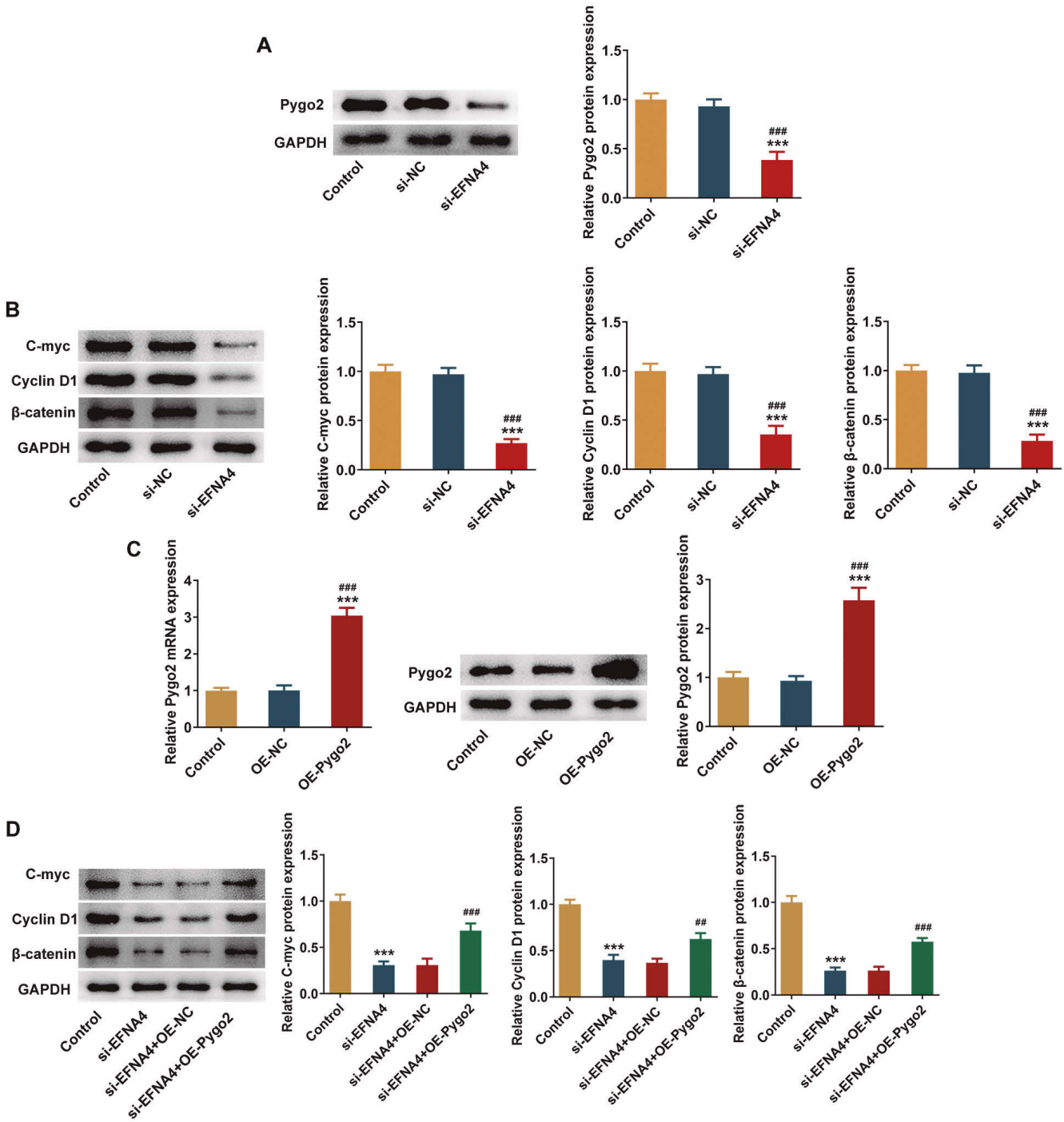

Fig. 5. Deletion of EFNA4 inactivates Pygo2/Wnt/β-catenin signaling. **A.** Western blot analysis of Pygo2 protein expression. ***p<0.001 vs. Control. ###p<0.001 vs. si-NC. **B.** Western blot analysis of Wnt/β-catenin signaling-associated proteins. ***p<0.001 vs. Control. ###p<0.001 vs. si-NC. **C.** Transfection efficacy of Pygo2 overexpression plasmids by RT-qPCR and western blot. ***p<0.001 vs. Control. ###p<0.001 vs. OE-NC. **D.** Western blot analysis of Wnt/β-catenin signaling-associated proteins. ***p<0.001 vs. Control. ##p<0.01, ###p<0.001 vs. si-EFNA4+OE-NC.

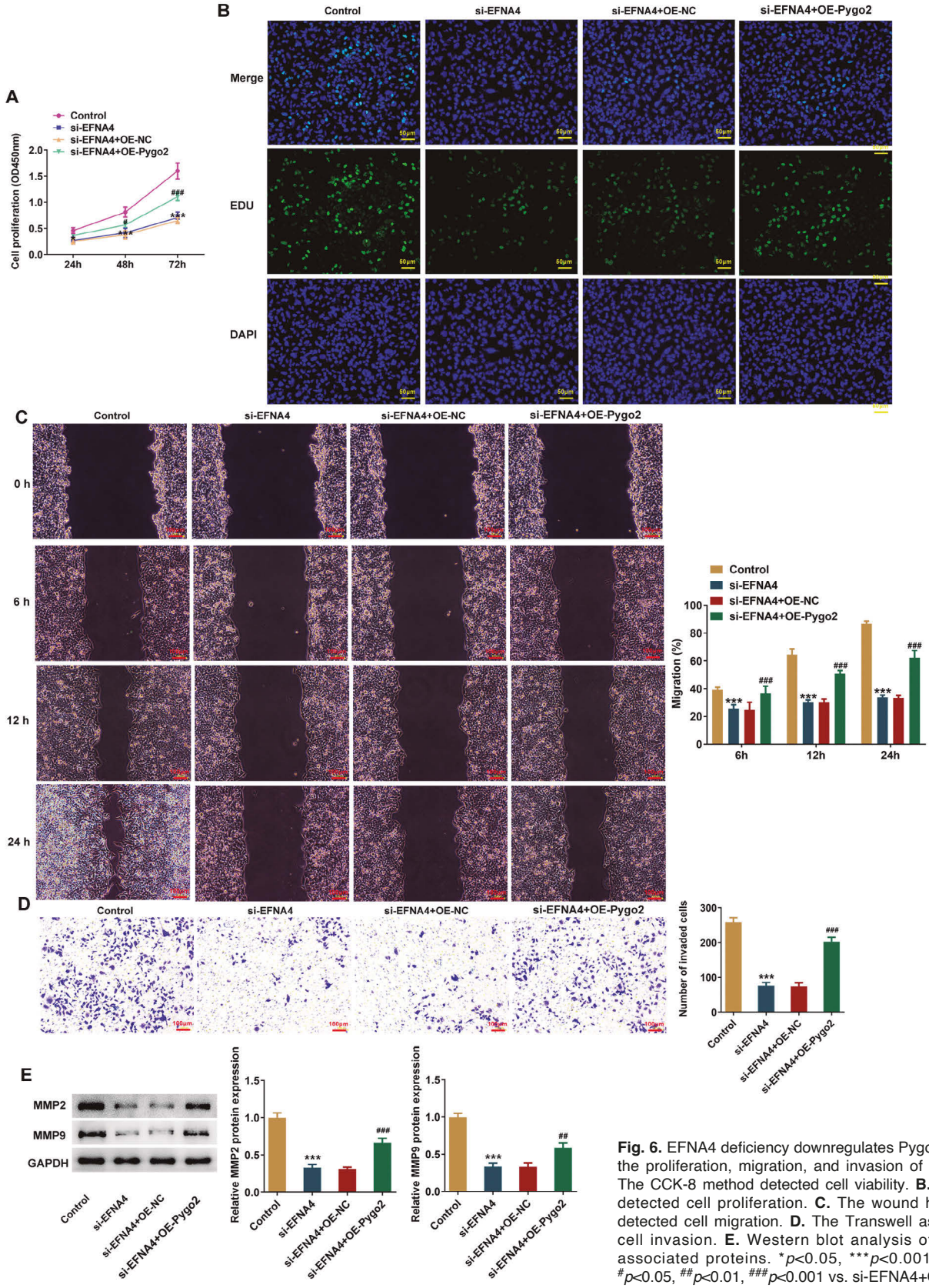

Fig. 6. EFNA4 deficiency downregulates Pygo2 to suppress the proliferation, migration, and invasion of AGS cells. **A.** The CCK-8 method detected cell viability. **B.** EdU staining detected cell proliferation. **C.** The wound healing assay detected cell migration. **D.** The Transwell assay detected cell invasion. **E.** Western blot analysis of metastasis-associated proteins. $^{*}p<0.05$, $^{***}p<0.001$ vs. Control. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. si-EFNA4+OE-NC.

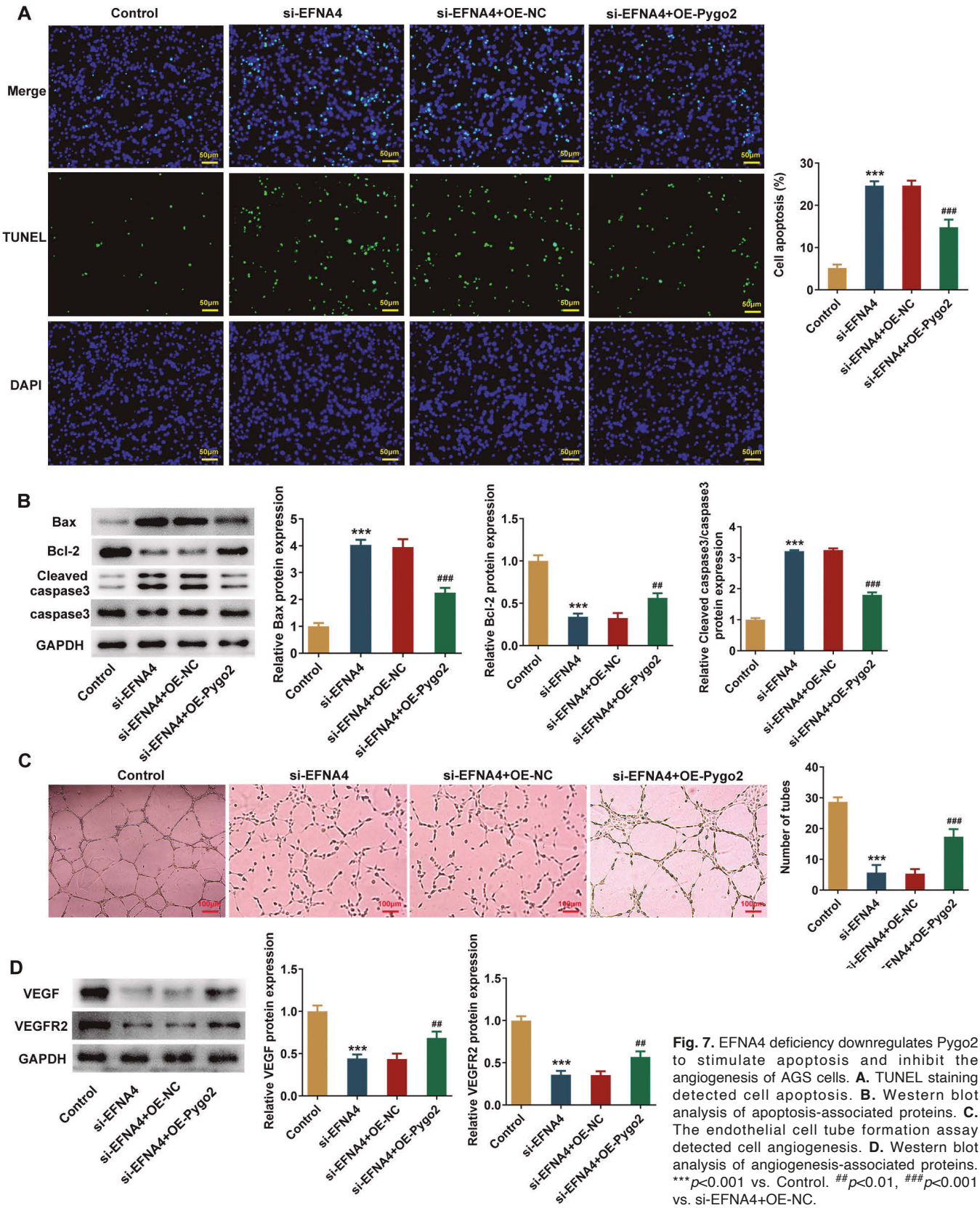

Fig. 7. EFNA4 deficiency downregulates Pygo2 to stimulate apoptosis and inhibit the angiogenesis of AGS cells. **A.** TUNEL staining detected cell apoptosis. **B.** Western blot analysis of apoptosis-associated proteins. **C.** The endothelial cell tube formation assay detected cell angiogenesis. **D.** Western blot analysis of angiogenesis-associated proteins. $^{***}p<0.001$ vs. Control. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. si-EFNA4+OE-NC.

EFNA4 deficiency downregulates Pygo2 to stimulate apoptosis and inhibit the angiogenesis of AGS cells

Conversely, the strengthened cell apoptotic rate caused by the knockdown of EFNA4, accompanied by upregulated Bax and Cleaved caspase-3/Caspase-3 expression, and the downregulated Bcl-2 expression, were partially reversed after Pygo2 was overexpressed

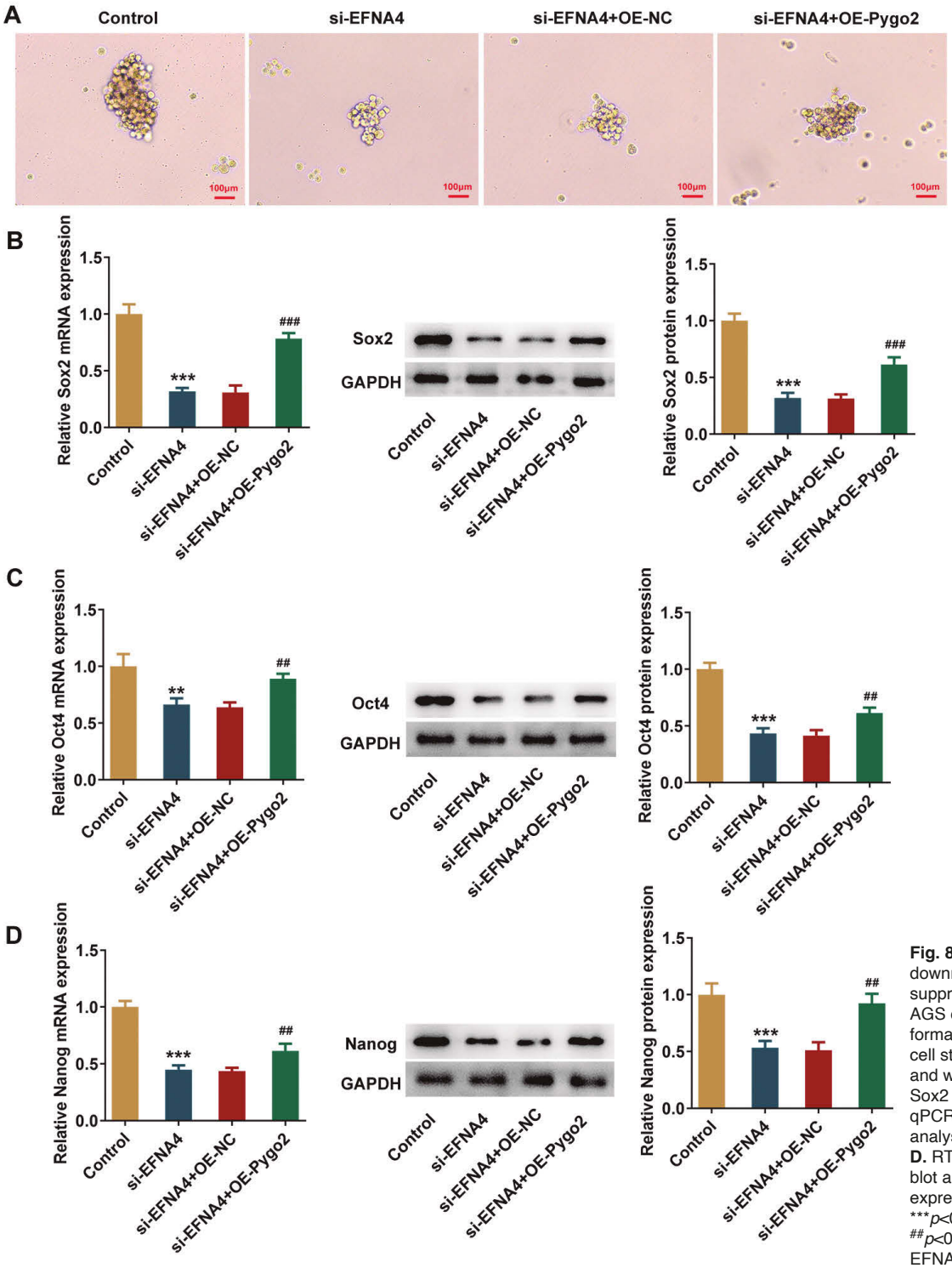

Fig. 8. EFNA4 deficiency downregulates Pygo2 to suppress the stemness of AGS cells. **A.** The sphere formation assay detected cell stemness. **B.** RT-qPCR and western blot analysis of Sox2 expression. **C.** RT-qPCR and western blot analysis of Oct4 expression. **D.** RT-qPCR and western blot analysis of Nanog expression. $^{**}p<0.01$, $^{***}p<0.001$ vs. Control. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. si-EFNA4+OE-NC.

(Fig. 7A,B). Meanwhile, the elevation of Pygo2 also restored the downward trend of cell angiogenesis, VEGF, and VEGFR1 expression, due to EFNA4 silencing in AGS cells (Fig. 7C,D).

EFNA4 deficiency downregulates Pygo2 to suppress the stemness of AGS cells

Simultaneously, the sphere formation assay, RT-qPCR, as well as western blot analysis, showed that, relative to the Control group, cell stemness was repressed and Nanog, Sox2, and Oct4 expression were all lowered in the si-EFNA4 group. However, cell stemness was accelerated and Nanog, Sox2, and Oct4 expression were elevated in the si-EFNA4+OE-Pygo2 group compared with the si-EFNA4+OE-NC group (Fig. 8A-D).

Discussion

The pathogenic and metastatic mechanism of gastric cancer is complex and multifactorial involving genetic alterations (Biagioni et al., 2019). Current data have established that angiogenesis and stemness are responsible for the development and metastasis of gastric cancer (Nienhüser and Schmidt, 2017; Rao et al., 2022). The present study aimed to provide insights into angiogenesis and stemness-associated targets to inhibit the progression of gastric cancer. Here, this work confirmed that EFNA4 was a contributor to cell proliferation, migration, invasion, angiogenesis, as well as stemness, in gastric cancer, which functioned through mediating Pygo2/Wnt signaling.

Existing research revealed that EFNA4 expression is activated in lung adenocarcinoma, hepatocellular carcinoma, oral squamous cell carcinoma, and sonic hedgehog medulloblastoma (Chen et al., 2021; Lin et al., 2021; Yuan et al., 2022; Zhao et al., 2022). EFNA4 was speculated to be highly expressed in STAD tissues according to the GSE103236 dataset from the GEO database. The raised EFNA4 expression in gastric cancer cells was also evidenced here. Simultaneously, growing proof has proposed that EFNA4 may act as a tumor promoter by facilitating cell proliferation, migration, invasion, and adhesion (Chen et al., 2021; Lin et al., 2021; Yuan et al., 2022; Zhao et al., 2022). Overexpression of metastasis-related MMP2 and MMP9 expression has been noticed in invasive and metastatic cases of patients with gastric cancer (Dong et al., 2020). In keeping with previous studies, the viability, proliferation, migration, and invasion of AGS cells were repressed after EFNA4 was depleted, which was concomitant with the downregulated MMP2 and MMP9 expression. In addition, the stimulatory role of EFNA4 silencing in the apoptosis of AGS cells was also discovered through investigation, accompanied by the elevated pro-apoptotic Cleaved caspase-3 and Bax expression and reduced anti-apoptotic BCL2 expression.

Angiogenesis is defined as the formation of new blood vessels from preexisting vessels (Olejarz et al., 2020). As mentioned by Professor Folkman in the 1970s, angiogenesis, one of the hallmarks of cancer, is essential for tumor growth, and invasion; thus, inhibition of angiogenesis could be an effective strategy for tumor treatment (Oguntade et al., 2021). Substantial proof has elucidated that gastric cancer is recognized as a highly angiogenic cancer (Hsieh and Tsai, 2019). VEGF, a homodimer glycoprotein, is a pivotal mediator of tumor angiogenesis, presenting crucial pro-angiogenic properties (Melincovici et al., 2018). VEGFR2 is a cell-surface tyrosine kinase receptor of VEGF that mediates VEGF-induced angiogenesis, vascular permeability, and vascular remodeling (Karali et al., 2014). The binding of VEGF to VEGFR2 causes receptor autophosphorylation and initiates a string of signaling cascades, resulting in cell proliferation, migration, and survival (Simons et al., 2016). Noticeably, in comparison with the research revealing that EFNA4 knockdown can protect against angiogenesis and deplete VEGF and VEGFR2 expression in hepatocellular carcinoma (Yuan et al., 2022), our experimental data delineated that EFNA4 deficiency decreased the number of tubes and VEGF and VEGFR2 expression in AGS cells, suggesting the anti-angiogenic activity of EFNA4 interference in gastric cancer cells.

Cancer stem cells (CSCs) are referred to as a subpopulation of tumor cells with stemness characteristics (Nassar and Blanpain, 2016). Dependent on the capacity to initiate tumor growth and sustain self-renewal, as well as differentiation, CSCs are deemed as predominant contributors to the drug resistance, initiation, and progression of gastric cancer (Rao et al., 2022). Moreover, Chen et al. reported that EFNA4 accelerates the spheroid formation of oral squamous cell carcinoma cells by upregulating Nanog expression (Chen et al., 2021). Nanog, Sox2, and Oct4, major transcription factors related to stem cell self-renewal and differentiation, have been positively involved in the pathogenesis of gastric cancer (Basati et al., 2020). Our study also demonstrated that EFNA4 deletion decreased the size and number of spheres formed, and downregulated Nanog, Sox2, and Oct4 expression in gastric cancer cells.

Previous evidence disclosed that EFNA4 deficiency may function as a tumor suppressor in hepatocellular carcinoma via reducing Pygo2 expression (Yuan et al., 2022). Accumulating proof has demonstrated that Pygo2 combines with free β−catenin to cause abnormal activation of downstream target genes, including c-Myc and cyclin D1 (Andrews and Kao, 2016). Consequently, Pygo2 has been confirmed as a newly identified component of the Wnt/β-catenin pathway in multiple human malignancies (Talla and Brembeck, 2016; Zhang et al., 2016; Zhou et al., 2020). Additionally, EFN/EPH signaling pathway networks are related to the Wnt signaling pathway during embryogenesis, tissue regeneration, and carcinogenesis (Yoon et al., 2023). As expected, EFNA4 may act as a positive modulator of the

Wnt/β-catenin pathway (Yuan et al., 2022). Consistently, knockdown of EFNA4 was discovered to block the Pygo2/Wnt/β-catenin pathway by inactivating Pygo2, C-myc, Cyclin D1, and β-catenin expression. Importantly, Pygo2 may regulate the drug resistance of gastric cancer cells (Zhang et al., 2021). Further, Wnt/β-catenin signaling has also been illustrated to participate in the carcinogenesis, metastasis, angiogenesis, and stemness of gastric cancer (Chiurillo, 2015; Pajuelo-Lozano et al., 2020; Zhang et al., 2023). Here, we proved that Pygo2 upregulation reversed the impacts of EFNA4 silencing on Wnt/β-catenin signaling, proliferation, migration, invasion, apoptosis, angiogenesis, and stemness in gastric cancer cells.

Conclusively, the absence of EFNA4 could exhibit potential antitumor activity in gastric cancer, which might be partially attributed to inactivation of Pygo2/Wnt/β-catenin signaling, representing that EFNA4 might be a prospective therapeutic target in gastric cancer. Considering the demonstrated oncogenic role of EFNA4 in gastric cancer in the present work, PF-06647263, a novel antibody-drug conjugate consisting of an anti-EFNA4 antibody linked to a calicheamicin payload, may also be applied to gastric cancer therapy since PF-06647263 has shown potent clinical efficacy against breast cancer and ovarian cancer (Damelin et al., 2015; Garrido-Laguna et al., 2019; Fraguas-Sánchez et al., 2022).

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