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Induction of Fibroblast Growth Factor Receptor 4 by *Helicobacter pylor*i via Signal Transducer and Activator of Transcription 3 With a Feedforward Activation Loop Involving Steroid Receptor Coactivator Signaling in Gastric Cancer

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Abstract

BACKGROUND & AIMS: *Helicobacter pylori* (*H pylori*) infection is the main risk factor for gastric cancer. The role of fibroblast growth factor receptors (FGRFs) in *H pylori*-mediated gastric

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Conflicts of interest

The authors disclose no conflicts.

Supplementary Material

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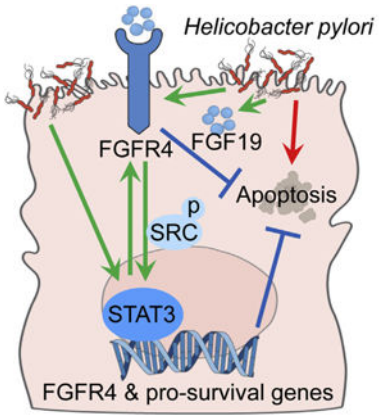
tumorigenesis remains largely unknown. This study investigated the molecular and mechanistic links between *H pylori*, inflammation, and *FGFR4* in gastric cancer.

METHODS: Cell lines, human and mouse gastric tissue samples, and gastric organoids models were implemented. Infection with *H pylori* was performed using in vitro and in vivo models. Western blot, real-time quantitative reverse-transcription polymerase chain reaction, flow cytometry, immunofluorescence, immunohistochemistry, chromatin immunoprecipitation, and luciferase reporter assays were used for molecular, mechanistic, and functional studies.

RESULTS: Analysis of FGFR family members using The Cancer Genome Atlas data, followed by validation, indicated that *FGFR4* messenger (m)RNA was the most significantly overexpressed member in human gastric cancer tissue samples ($P < .001$). We also detected high levels of *Fgfr4* mRNA and protein in gastric dysplasia and adenocarcinoma lesions in mouse models. Infection with J166, 7.13, and PMSS1 cytotoxin-associated gene A (CagA)+ *H pylori* strains induced *FGFR4* mRNA and protein expression in in vitro and in vivo models. This was associated with a concordant activation of signal transducer and activator of transcription 3 (STAT3). Analysis of the *FGFR4* promoter suggested several putative binding sites for STAT3. Using chromatin immunoprecipitation assay and an *FGFR*-promoter luciferase reporter containing putative STAT3 binding sites and their mutants, we confirmed a direct functional binding of STAT3 on the *FGFR4* promoter. Mechanistically, we also discovered a feedforward activation loop between FGFR4 and STAT3 where the fibroblast growth factor 19–FGFR4 axis played an essential role in activating STAT3 in a steroid receptor coactivator–dependent manner. Functionally, we found that FGFR4 protected against *H pylori*-induced DNA damage and cell death.

CONCLUSIONS: Our findings demonstrated a link between infection, inflammation, and FGFR4 activation, where a feedforward activation loop between FGFR4 and STAT3 is established via steroid receptor coactivator in response to *H pylori* infection. Given the relevance of FGFR4 to the etiology and biology of gastric cancer, we propose FGFR4 as a druggable molecular vulnerability that can be tested in patients with gastric cancer.

Graphical Abstract



Gastroenterology

Keywords

FGFR4; *H pylori* ; Gastric Cancer; STAT3; SRC

According to Global Cancer Statistics 2020, gastric cancer is the fifth most common cancer and the fourth leading cause of cancer-related death worldwide.[1] In addition, patients with gastric cancer are often diagnosed at late stages with distant metastasis and chemoresistance.[2,3] According to the National Cancer Institute's Surveillance, Epidemiology, and End Results database, the combined 5-year survival rates are ~30% for patients with gastric cancer.[4] Therefore, there is a critical need for a better understanding of the biology mechanisms driving gastric tumorigenesis to improve the current diagnostic, prognostic, and therapeutic approaches.

Helicobacter pylori (*H pylori*) infection is the most common risk factor for gastric cancer, affecting more than half of the world's population.[5] The World Health Organization has classified *H pylori* as a class I carcinogen.[6] *H pylori* infection is strongly associated with chronic gastritis, peptic ulcer, and mucosa-associated lymphoid tissue lymphoma. Furthermore, chronic infection can lead to a stepwise progression from gastric atrophy, intestinal metaplasia, dysplasia, and finally to adenocarcinoma, known as the Correa's cascade of gastric carcinogenesis.[7] In addition, chronic *H pylori* infection can lead to emerging populations of tumorigenic gastric cells that can survive and coexist with a high level of DNA damage.[8,9] These abnormal tumorigenic gastric epithelial cells mediate progression to gastric cancer.[10]

During *H pylori* infection, multiple cytokines, such as interferons and interleukins, play vital roles in regulating the immune and inflammatory responses. For example, signal transducer and activator of transcription 3 (STAT3) and its downstream signaling targets[11] are activated during gastric carcinogenesis, playing an important role in cancer initiation, progression, and poor patient survival.[12,13] Activation of STAT3 is a multistep process starting from a transient activation in normal cells during early steps of *H pylori* infection to a persistent phosphorylation/activation state in neoplastic gastric cells. The upstream tyrosine kinases such as epidermal growth factor receptor, steroid receptor coactivator (SRC), and Janus kinase, are crucial mediators of STAT3 activation in response to infection and genotoxic stress stimuli.[14–16]

Fibroblast growth factor receptors (FGFRs) are a gene family of transmembrane tyrosine kinase receptors that activate important downstream signaling pathways.[17,18] Some known canonical downstream signaling pathways of FGFRs include RAS–mitogen-activated protein kinase, phosphoinositide 3-kinases–AKT, and phospholipase-Cγ–Ca^{2+}–protein kinase C.[19,20] The patterns of FGFR and FGF ligand expression underlie context-dependent biological and mechanistic functions during the initiation and progression of cancer. Among FGFRs, FGFR4 was reported to promote cancer cell proliferation, invasion, survival, and chemoresistance in multiple cancer types.[21,22] The fibroblast growth factor 19 (FGF19)-FGFR4–dependent signaling has been reported as critical pathway in the development of hepatocellular carcinomas.[23]

Although a number of studies show overexpression of FGFR family members in gastric cancer,[24,25] the role of *H pylori* remains incompletely understood. We therefore performed a systematic analysis of FGFR family members in gastric cancer data sets in the present study. Based on the findings, we focused on investigating the link between *H pylori* infection,

inflammation, and activation of *FGFR4*. As a result we discovered FGFR4 as a direct transcription target of STAT3, where SRC mediated a positive feedforward loop between FGFR4 and STAT3 in response to *H pylori* infection.

Materials and Methods

Animals

All animal experiments were approved by the University of Miami Institutional Animal Care and Use Committees (IACUC #20–110). C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and challenged with *H pylori* or *Brucella* broth (control) using orogastric gavage. Mice were assigned to 2 groups: uninfected control or infected with the mouse-adapted wild-type (WT) *H pylori* PMSS1 strain (10^9 colony forming units/mouse). We euthanized mice 1 week, 2 weeks, or 4 months after infection. The trefoil factor 1–knockout (TFF1-KO) mice[26] and WT mice were used to investigate the association between *FGFR4* and *H pylori*-mediated gastric tumorigenesis. We also used paraffin-embedded stomach tissues from the $GP130^{Y757F/Y757F}$ ($GP130^{F/F}$) mouse model.[27]

Three-Dimensional Organoid Cultures

Three-dimensional organoid cultures were established from mouse and human stomachs, following the protocol by Leelatian et al.[28] Gastric tissue samples from the antropyloric regions from human and 4- to 8-week-old mice were subjected to dissociation. Briefly, iced Hank's balanced salt solution was used to wash the tissues, followed by cutting into small pieces and incubation in prewarmed gland digestion media containing Dulbecco's modified Eagle medium supplemented with 20 mmol/L HEPES buffer (Corning, Corning, NY), 0.2% fetal bovine serum (FBS), 1% penicillin/streptomycin (Gibco, Waltham, MA), 50 μg/mL gentamicin, and 1 mg/mL of collagenase A (Sigma-Aldrich, St Louis, MO). The tissues were agitated by gentle inversion for 40 minutes at 37°C and centrifuged 5 minutes at 100*g*. The supernatant containing connective tissue was aspirated, and the cell pellet was resuspended in 10 mL of prewarmed Gland Rinse Media containing DMEM/F12 (Gibco), 1% penicillin/streptomycin, 50 μg/mL gentamicin, and 0.5 mmol/L dithiothreitol (Research Products International, Theodore, AL). The suspension was centrifuged, and the pellet was resuspended in prewarmed Trypsin-EDTA (Sigma-Aldrich). Trypsin was quenched with 2 mL of PBS supplemented with FBS. The suspension was sequentially passed twice through 40-μm cell strainers (Becton Dickson, Franklin Lakes, NJ), and then centrifuged at 150*g* for 5 minutes.

The pelleted single cells were embedded in an extracellular matrix hydrogel (Trevigen, Inc, Gaithersburg, MD) supplemented with IntestiCult Organoid Growth Medium (StemCell Technologies, Vancouver, BC, Canada), which is the suitable culture medium for building gastric organoid models. The medium contained several growth factors vital for the differentiation and growth of gastric organoid models, including noggin (100 ng/mL), Wnt-3A (100 ng/mL), epidermal growth factor (50 ng/mL), FGF10 (100 ng/mL) (Peprotech Inc, Rocky Hill, NY), R-spondin (1 μg/mL) (R&D Systems, Minneapolis, MN), and gastrin (10 nmol/L) (Sigma-Aldrich). Glands matured into organoids by the third day and were passaged every 12 days. Organoids were removed from Matrigel (Corning) using a gentle

cell dissociation reagent (StemCell Technologies) and processed for standard formalin-fixed embedded tissues with HistoGel (VWR, Radnor, PA).

Fibroblast Growth Factor Receptor 4 Overexpression and Small Interfering RNA Knockdown

The pHAGE-FGFR4 overexpressing plasmid was purchased from Addgene (Watertown, MA). The pcDNA3-lacZ vector was a kind gift from Dr Michael K. Cooper (Vanderbilt University). We transfected AGS and MKN-28 cells transiently with 1 μg of FGFR4 plasmid or control vector (Abmgood, Richmond, BC, Canada) using the PolyJet transfection reagent (SignaGen Laboratories, Frederick, MD). At 48 hours after transfection, cells were treated with 2.5 μmol/L dasatinib (Selleckchem, Houston, TX) or transfected with SRC or control small interfering (si) RNA (control: 40 nmol/L, time point: 48 hours) using LipoJet (SignaGen Laboratories, Rockville, MD). Control siRNA (universal negative control) was purchased from Sigma-Aldrich; FGFR4 siRNA-1 (ID: 1412) was obtained from Thermo Fisher Scientific (Waltham, MA), and FGFR4–2 siRNA-2 (sc-35368) and SRC siRNA (sc-29228) were obtained from Santa Cruz Biotechnology (Dallas, TX). Western blots and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis validated the transfection efficiency.

Helicobacter pylori *Strains*—The WT cytotoxin-associated gene A (CagA) *H pylori* strains (7.13, J166, and the rodent-adapted PMSS1) were obtained from Dr Richard Peek Jr (Vanderbilt University Medical Center). Trypticase soy agar with 5% sheep blood agar plates (BD Biosciences, Bedford, MA) was used to culture and passage *H pylori* bacteria. *H pylori* were grown in *Brucella* broth (BD Biosciences) supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA) for 16 hours at 37°C with 5% CO_2 to amplify and activate their virulence. Epithelial cells were infected by *H pylori* at a multiplicity of infection of 100:1 at different time points, following standard protocols.[29,30]

Results

Fibroblast Growth Factor Receptor 4 Is Upregulated in Gastric Cancer Tissue Samples

To investigate the levels of the FGFR family members in human gastric cancer, we analyzed the expression of the 4 FGFRs (FGFR1, 2, 3, and 4) using The Cancer Genome Atlas (TCGA) database with 35 normal gastric and 415 gastric cancer tissue samples. This analysis indicated that FGFR4 was the only significantly overexpressed member in gastric cancer tissues ($P < .001$) (Figure 1A). To validate these findings, we performed qRT-PCR using 94 gastric cancer and 54 normal gastric tissue samples. These included 45 paired normal gastric and gastric cancer tissues (Supplementary Tables 1, 2, and 3). Our data indicated that *FGFR4* messenger RNA (mRNA) was significantly higher in gastric cancer than in normal gastric samples ($P < .001$) (Figure 1B). In addition, immunohistochemistry analysis of human tissues showed high levels of FGFR4 protein in gastric cancer tissue samples compared with normal samples ($P < .05$) (Figure 1C).

Next, we used the TFF1-KO mouse model, known to develop progressive neoplastic lesions that include low-grade dysplasia, high-grade dysplasia, and invasive adenocarcinoma in

the antropyloric glandular region of the mouse stomach.[26] Similar to humans, analysis of stomach tissues from the TFF1-KO mouse demonstrated significant overexpression of *Fgfr4* mRNA in dysplastic and adenocarcinoma lesions ($P < .05$) (Figure 1D). These data were confirmed by immunohistochemistry analysis that showed high levels of FGFR4 in neoplastic lesions (Figure 1E). The results from humans and mice suggest that FGFR4 could play an important role in gastric tumorigenesis.

To determine the prognostic significance of *FGFR4* in gastric cancer, we analyzed the TCGA data and used the median *FGFR4* mRNA expression value to divide patients into *FGFR4*-high and *FGFR4*-low groups. The Kaplan-Meier plotter survival analysis indicated that patients with gastric cancer with lower *FGFR4* expression had significantly longer overall survival (low: 70.2 months, high: 35.2 months) and disease-free survival (low: 57.2 months, high: 25.3 months) (Figure 1F). Furthermore, we analyzed the *FGFR4* expression among different gastric cancer types according to Lauren's classification.[31] Our data identified high *FGFR4* expression as a poor prognostic indicator in patients with intestinal-type (low: 99.4 months, high: 38.2 months) (Figure 1G), but not with patients with diffuse- or mixed-type gastric cancer (Supplementary Figure 1A).

Fibroblast Growth Factor Receptor 4 Expression and Signal Transducer and Activator of Transcription 3 Activation Are Induced by Helicobacter pylori *Infection*

H pylori infection is the main risk factor of gastric cancer.[32] We hypothesized that infection with *H pylori* played a role in FGFR4 overexpression. Therefore, we analyzed the TCGA data using the gene set enrichment analysis (GSEA) of *H pylori* molecular signature (https://www.gsea-msigdb.org/gsea/msigdb/cards/KEGG_EPITHELIAL_CELL_SIGNALING_IN_HELICOBACTER_PYLORI_INFECTION). This analysis indicated that the *H pylori* molecular signature was significantly enriched in the *FGFR4*-high compared with *FGFR4*-low gastric cancer samples ($P < .001$) (Figure 2A). Based on these results, we infected AGS and MKN28 cells with *H pylori* (J166 and 7.13 strains) to determine the causal relationship. The qRT-PCR data indicated that *FGFR4* mRNA expression was the most significantly induced FGFR family member at 6 and 24 hours ($P < .001$) (Figure 2B and Supplementary Figure 1B and C). Changes in the mRNA expression of other FGFR family members were modest and significantly lower than *FGFR4* (Supplementary Figure 1B).

To confirm our findings in vivo, we infected mice with the PMSS1 mouse-adapted *H pylori* strain and examined *Fgfrs* expression levels. Our data indicated that *Fgfr1* and *Fgfr4* mRNAs were induced after 1 week of infection with PMSS1 *H pylori*, but not *Fgfr2* or *Fgfr3*. However, at 2 weeks of infection, only *Fgfr4* mRNA was consistently and significantly induced ($P < .05$) (Supplementary Figure 1D). These results suggested that *H pylori* infection played an important role in the induction of *FGFR4*.

H pylori infection is associated with a strong oncogenic inflammatory response during the gastric carcinogenesis cascade. We and others have shown STAT3 as an important driver of gastric tumorigenesis.[13,33] Western blot analysis showed an increase in total and phosphorylated levels of FGFR4 and STAT3 after *H pylori* infection of AGS and MKN28 cells (Figure 2C and Supplementary Figure 1E). The same findings were obtained in

vivo after infection of mice with the PMSS1 mouse-adapted *H pylori* strain (Figure 2D) compared with the uninfected control. Similar results were achieved using normal gastric epithelial cells (GES-1) (Supplementary Figure 2A).

In addition, immunofluorescence staining demonstrated a significant increase in FGFR4 expression and phosphorylated (p)-STAT3 nuclear fluorescence intensity (at least 100 cells were counted) after *H pylori* infection in AGS cells ($P < .001$) (Supplementary Figure 2B and C). To validate the in vitro cells data, we determined the protein levels of p-STAT3 and FGFR4 using glandular antropyloric stomach tissues from mice (in vivo model). Western blot analysis indicated that mice infected with *H pylori* PMSS1 (2 weeks) showed a significant increase of p-FGFR4, FGFR4, and p-STAT3 protein levels (Figure 2D) compared with noninfected control mice.

Immunofluorescence analysis confirmed these results, showing an increase in FGFR4 and nuclear localization of p-STAT3 in the antropyloric regions of gastric tissue from *H pylori*-infected mice compared with noninfected mice ($P < .001$) (Figure 2E). Similarly, the immunofluorescence staining on 3-dimensional organoids derived from normal human gastric tissues showed a significant increase in nuclear localization of p-STAT3 ($P < .001$) (Figure 2F) and a remarkable increase of FGFR4 after *H pylori* infection compared with noninfected human organoids (Figure 2F). These data indicated that *H pylori* induced high levels of FGFR4 with activation of STAT3 in vitro, in vivo, and ex vivo.

Signal Transducer and Activator of Transcription 3 Activation Regulates Fibroblast Growth Factor Receptor 4 In Vitro and In Vivo

Based on our results, we hypothesized that STAT3 can be involved in the regulation of FGFR4. We and others have previously shown activation of STAT3 in several gastric cancer mouse models, including the TFF1-KO[13] and GP130$^{Y757F/Y757F}$.[27] Therefore, we used these mouse models to determine levels of FGFR4 and p-STAT3. Immunofluorescence analysis demonstrated high levels of FGFR4 with p-STAT3 nuclear localization in gastric tissues of 1.5-month-old and 3.5-month-old GP130$^{Y757F/Y757F}$ mice compared with WT C57/BL6 mice (Figure 3A). We also confirmed these data using the TFF1-KO mouse model, where the immunofluorescence analysis showed an increase in the levels of FGFR4 and nuclear p-STAT3 in the antropyloric dysplastic lesions compared with normal gastric tissues (Figure 3B).

Using interleukin 6 (IL6) stimulation to activate STAT3 in AGS and MKN-28 cells led to an increase in the mRNA and protein levels of FGFR4 at 30 and 120 minutes. We did not detect changes at 60 minutes, suggesting the presence of an early response to *H pylori* (30 minutes), followed by cyclic changes (120 minutes) to prevent hyperactivation of STAT3 (Figure 3C and Supplementary Figure 2D). Using the AGS and MKN28 gastric cancer cell lines, we found the use of the STAT3 inhibitor, napabucasin,[34] decreased p-STAT3 (Y705) levels and abrogated the *H pylori*-mediated increase in FGFR4 at both mRNA and protein levels (Figure 3D and E and Supplementary Figure 2E). Under the same conditions, napabucasin reversed the *H pylori*-mediated increase in nuclear STAT3 and FGFR4 immunostaining (Figure 3F).

To confirm these data, 2 groups of WT mice were infected with PMSS1 *H pylori*. After 2 weeks, 1 group of the infected mice was intraperitoneally injected with napabucasin (40 mg/kg) every other day for 1 week. After the last dose, all animals were humanely killed, including the uninfected control mice. The qRT-PCR data demonstrated a significant increase of *Fgfr4* mRNA expression in mouse gastric tissues after PMSS1 *H pylori* infection ($P < .01$) (Figure 3G). This increase was significantly abolished after treatment with napabucasin ($P < .01$) (Figure 3G). Western blot analysis confirmed the qRT-PCR data and showed an increase of FGFR4 protein level in the PMSS1-infected mice compared with uninfected WT mice. The treatment with napabucasin reduced the FGFR4 protein levels in gastric tissue of the infected mice (Figure 3H). The effect of napabucasin was confirmed by reduced p-STAT3 protein levels (Figure 3H). Taken together, these results suggested an upstream regulation of *FGFR4* mRNA levels by STAT3.

Fibroblast Growth Factor Receptor 4 Expression Is Induced via Direct Binding of Signal Transducer and Activator of Transcription 3 on the Fibroblast Growth Factor Receptor 4 Promoter

Next, we analyzed the FGFR4 promoter using 2 databases, the JASPAR (http://jaspar.genereg.net) and the Animal Transcription Factor DataBase (AnimalTFDB, version 3.0; http://bioinfo.life.hust.edu.cn/AnimalTFDB/). This analysis demonstrated 9 possible STAT3 binding sites on the FGFR4 promoter region (Figure 4A, *blue bars*). Using an FGFR4 luciferase promoter-reporter assay containing these STAT3 binding sites, we detected an increase in the luciferase activity in AGS and MKN-28 cells after *H pylori* infection (8 hours) or IL6 treatment (100 ng/mL, 6 hours; $P < .05$) (Figure 4B and C). Inhibition of STAT3 by napabucasin abrogated the luciferase induction under these conditions, further supporting the role of STAT3 in activation of the FGFR4 promoter ($P < .05$) (Figure 4B and C). To confirm the STAT3 binding on the FGFR4 promoter region, we designed 6 pairs of primers (P1–P6) that cover binding sites for chromatin immunoprecipitation (ChIP) assay (Figure 4A, P1 to P6). qRT-PCR analysis using STAT3-conjugated DNA samples showed amplifications with P2 and P4 primers, suggesting these as candidate binding sites.

After treatment with IL6, the ChIP assay demonstrated a significant increase in the amplification of the fragments containing P2 or P4 binding sites compared with PBS treatment ($P < .01$) (Figure 4D). These results indicated IL6 induced FGFR4 expression through direct STAT3 binding on the *FGFR4* gene promoter, possibly through binding sites in the P2 and P4 regions.

Fibroblast Growth Factor 19–Fibroblast Growth Factor Receptor 4 Axis Promotes a Feedforward Activation Loop of Signal Transducer and Activator of Transcription 3 via Steroid Receptor Coactivator Activation

Several reports have indicated that activation of FGFR4 is mediated by several ligands such as FGF1, FGF3, and *FGF19* in cancer.[35,36] Using qRT-PCR, we analyzed the expression of these ligands in normal and cancerous gastric tissue samples. *FGF19* mRNA expression was significantly higher in gastric cancer tissues than in normal gastric glands ($P < .05$) (Figure 5A). The TCGA database analysis confirmed our findings and showed significantly

higher levels of *FGF19* in gastric cancers compared with normal tissues ($P < .001$) (Figure 5B). Furthermore, by analyzing survival data of patients with gastric cancer from the TCGA database, we found that patients with lower *FGF19* expression had significantly longer overall survival and disease-free survival (Figure 5C). A combined survival analysis of *FGFR4* and *FGF19* in gastric cancer patients indicated that high *FGFR4*/*FGF19* expression levels are associated with worse overall survival (low: 93.2 months, high: 31.2 months) (Figure 5D) or disease-free survival (low: 73.7 months, high: 23.2 months) (Figure 5D) compared with low *FGFR4*/*FGF19* expression. Analysis of *Fgf15*, the mouse ortholog for human FGF19, demonstrated a significant and progressive increase of *Fgf15* expression level across stages of gastric tumorigenesis (low-grade dysplasia → high-grade dysplasia/cancer) in the TFF1-KO mice ($P < .05$) (Figure 5E).

Next, we analyzed the effect of *H pylori* on the expression of *Fgf15* in WT mice. qRT-PCR data demonstrated significant induction of the *Fgf15* mRNA expression level after 1 and 2 weeks of PMSS1 *H pylori* infection ($P < .01$) (Figure 5F). We also investigated the long-term infection and found an increase of *Fgfr4* and *Fgfr15* at 1 and 4 months of *H pylori* infection (Supplementary Figure 3A and B, respectively). Using AGS and MKN-28 gastric cells, we detected a significant increase in *FGF19* after infection with *H pylori* (7.13 and J166) for 3 and 6 hours ($P < .01$) (Figure 5G and Supplementary Figure 3C). These data collectively indicate the presence of high *FGF19*/*Fgf15* in humans and mice and demonstrate their induction in response to *H pylori* infection.

Earlier studies have shown that canonical activation of FGFR4 by its ligands, such as FGF19, mediate STAT3 activation.[37,38] In the context of our findings, this would suggest the presence of a feedforward loop between FGFR4 and STAT3 in gastric cancer cells. We therefore investigated the effect of FGF19 on canonical activation of FGFR4 and its downstream effectors such as STAT3 and extracellular signal–regulated kinase. Indeed, the use of FGF19 treatment (range, 100–400 ng/mL) led to an increase in the levels of p-FGFR4 (Y642), p-STAT3 (Y705), p-SRC (Y416), and p-extracellular signal–regulated kinase (T202/Y204) protein levels in AGS and MKN28 cells (Supplementary Figure 4A). Of note, the FGFR4 knockdown reversed these effects (Figure 5H and Supplementary Figure 4B).

To confirm these data, we established human normal gastric organoids transfected with control or FGFR4 short hairpin RNA. After treatment with FGF19 (200 ng/mL), the organoids with control short hairpin RNA showed an increase of FGFR4 and nuclear p-STAT3 staining at 4 hours and 24 hours. The knockdown of FGFR4 in these organoids diminished the levels of nuclear p-STAT3 staining (Supplementary Figure 4C).

We next investigated whether *H pylori*-induced expression of FGFR4 and activation of STAT3 signaling is dependent on FGF19. We therefore used *H pylori* infection with and without FGF19 antibody neutralization. Conditioned media from cells infected with 7.13 *H pylori* strain were used to culture MKN28 and AGS, which were treated or not with FGF19 neutralizing antibody. Cells incubated with the conditioned media without infection were used as a control. Western blot analysis indicated an increase of secreted FGF19 after *H pylori* infection in MKN28 (Figure 5I) and AGS (Supplementary Figure 4D). This increase was followed by an increase in p-FGFR4 (Y642), p-STAT3 (Y705), and p-SRC

(Y416). Blockade of FGF19 using a neutralizing antibody reversed the aforementioned *H pylori*-mediated signaling effects. The reduction of free FGF19 protein was confirmed in the supernatant of the conditioned media in both MKN-28 (Figure 5I) and AGS cells (Supplementary Figure 4D). Collectively, these data suggested that STAT3 played a role in the transcription induction of FGFR4, whereas the FGF19 promoted phosphorylation/activation of FGFR4 to drive a feedforward loop with activation of STAT3 and SRC.

Fibroblast Growth Factor Receptor 4–Steroid Receptor Coactivator Axis Is Crucial for Helicobacter pylori*-Induced Activation of Signal Transducer and Activator of Transcription 3 Signaling*

Our results indicated that FGF19 induced phosphorylation of FGFR4, STAT3, and SRC. Using the TCGA database analysis, we detected a positive correlation between *SRC* and *FGFR4* expression among gastric cancer tissue samples (Figure 6A). GSEA was performed using the TCGA data to determine the correlation between FGFR4 and SRC and STAT3 molecular signatures. The correlation between high *FGFR4* expression and the 2 selected gene sets from the Molecular Signatures Database (a collection of annotated gene sets for use with GSEA software, http://www.gsea-msigdb.org/gsea/msigdb/index.jsp) was performed. In this database, high gene expression signatures were identified, reflecting the activation status of SRC or downstream STAT3 pathways in the TCGA database (Figure 6B and C).

We then hypothesized that FGFR4 is upstream of SRC signaling in conditions of *H pylori* infection. Interestingly, the increase in p-SRC as well as p-STAT3 after *H pylori* infection was abolished after FGFR4 knockdown in both AGS and MKN-28 cells (Figure 6D). Similar results were found using normal gastric epithelial GES-1 cells (Figure 6E). Of note, SRC inhibition with dasatinib, a small molecule-inhibitor of SRC-family protein-tyrosine kinases, reduced p-SRC and p-STAT3 levels (Supplementary Figure 5A). At the same time, SRC knockdown or its inhibition with dasatinib abrogated the *H pylori*-mediated induction of p-SRC and p-STAT3 protein levels (Figure 6F and G). The observed reduction in p-STAT3 level was associated with a decrease in FGFR4, consistent with our results showing induction of FGFR4 by STAT3 activation. Overexpression of FGFR4 in conditions of SRC knockdown or its inhibition with dasatinib reversed phosphorylation and activation of STAT3 by FGFR4 (Supplementary Figure 5B and C). Collectively, these results confirmed the role of SRC in the activation of STAT3, suggesting the presence of a feedforward loop between FGFR4 and STAT3 where SRC mediates activation of STAT3 by FGFR4.

Fibroblast Growth Factor Receptor 4 Protects Gastric Cancer Cell Survival During Helicobacter pylori *Infection*

FGFR4 has been shown to promote cell survival in stress conditions.[39] Our analysis of *H pylori* infection demonstrated an increase in the levels of cleaved poly (adenosine-5′-diphosphate–ribose) polymerase and cleaved caspase 3 protein expression in conditions of FGFR4 knockdown compared with control siRNA (Figure 7A, Supplementary Figure 6A). Using the ApoTox-Glo assay (Promega, Madison, WI) to determine the cell viability and apoptosis (caspase 3/7), we found that FGFR4 knockdown led to a decrease in cell viability with an increase in caspase 3/7, compared with control (Figure 7B and C, Supplementary

Figure 6B and C). Consistent with Western blot data and the ApoTox-Glo analysis, the knockdown of FGFR4 in *H pylori*-infected cells significantly increased the percentage of apoptotic cells compared with control siRNA-transfected cells (Figure 7D, Supplementary Figure 6D).

To confirm the role of the FGFR4-STAT3 loop in cell survival, we investigated the levels of antiapoptotic genes such as *BCL-XL*, *BIRC5*, and *XIAP*, known to be transcription targets of STAT3. Using the TCGA database, we found that FGFR4 expression profiles positively correlated with *BCL2L1* (*BCL-XL*) and *BIRC5* in gastric cancer samples ($P < .001$) (Figure 7E). Our results were consistent with the TCGA data and indicated that infection with *H pylori* mediated a significant increase in the expression level of *BCL-XL*, *BIRC5*, and *XIAP* antiapoptotic genes; the expression levels of these genes were reversed after FGFR4 knockdown ($P < .01$) (Figure 7F and Supplementary Figure 6E). Taken together, our results confirmed the role of FGFR4-STAT3 signaling in promoting cell survival in conditions of *H pylori* infection.

Discussion

In this study, analysis of FGFR family members indicated that *FGFR4* was the most significantly up-regulated FGFR family member in human gastric cancer. Although a few studies of FGFR2 have been reported in gastric cancer,[40,41] the role of FGFR4 in relation to *H pylori* infection and gastric carcinogenesis remains incompletely understood. Using in vitro cells, organoids, and mouse models, we found that STAT3 mediates transcriptional up-regulation of *FGFR4* in response to *H pylori* infection. We also detected a feedforward loop between FGFR4 and STAT3, where FGFR4 also played a role in the activation of STAT3 via SRC.

STAT3 is a proinflammatory oncogenic transcription factor that mediates tumor initiation and progression in several cancer types, including gastric cancer.[13,14,42] Activation of STAT3 is an important signaling event detected in neoplastic stomach tissues from human and mouse models. The GP130$^{Y757F/Y757F}$ mouse model carries a mutated GP130 cytokine receptor signaling subunit that mediates hyperactivation of STAT3.[33] The TFF1-KO mouse model develops dysplastic and neoplastic gastric lesions in the background of chronic inflammation and activation of STAT3.[13] Our results demonstrated strong nuclear STAT3 and high levels of FGFR4 in the glandular gastric mucosa of both mouse models.

H pylori infection, affecting almost half of the world's population, is a critical risk factor for gastric tumorigenesis.[43] *H pylori* is a potent inducer of inflammatory signaling,[44] promoting a series of progressive histologic changes during gastric tumorigenesis, including chronic gastritis, low-grade dysplasia, high-grade dysplasia, and finally, adenocarcinoma.[45] We detected a significant increase in p-STAT3 in response to infection concordant with induction of FGFR4 in response to *H pylori*. Using human and mouse gastric tissue samples and in vitro models, we confirmed the relationship between *H pylori* infection and *FGFR4* expression. Similar to *H pylori* infection, IL6 treatment induced expression of *FGFR4*. The inhibition of STAT3 using napabucasin completely abrogated the expression of FGFR4. Our studies to determine a possible mechanistic link between STAT3 and FGFR4 uncovered

STAT3 binding sites on the *FGFR4* promoter. The ChIP assay confirmed the binding of STAT3 on the *FGFR4* promoter, supporting the role of STAT3 in the transcription regulation of *FGFR4*. These results illustrate the presence of a direct link between *H pylori* infection, inflammation/STAT3, and *FGFR4* upregulation in gastric cancer. It is possible that this regulatory signaling axis may exist in other cancer types showing *FGFR4* overexpression, such as cancers of the colon,[21] breast,[46] and liver.[47]

Earlier studies suggested STAT3 as one of the downstream effectors of FGFR4.[48,49] Notably, epidermal growth factor receptor, sharing some similarity in structure with FGFR4, can promote progression in lung cancer via the SRC/p21-activated kinase 1 pathway[50] and confer sorafenib resistance in hepatocellular cancer cells via a SRC/STAT3 signaling axis.[51] Based on these earlier observations, we hypothesized the presence of an uncharacterized feedforward loop between STAT3 and FGFR4, mediated by SRC.

To prove our hypothesis, we conducted experiments in conditions of canonical activation of FGFR4 by its ligands. We detected an increase in FGF19/FGF15 in human and mouse models. We found that FGF19 was the most significantly up-regulated ligand in response to *H pylori*. The human FGF19, FGF15 in rodents, binds selectively with high affinity to FGFR4.[52,53] We found that FGF19 treatment or *H pylori* infection induced activation of SRC in human gastric cancer cells. This effect was abrogated by FGFR4 knockdown. These findings provided an additional line of evidence supporting our hypothesis of a possible role of FGFR4 in regulating SRC.

We also detected activation of STAT3 in response to FGF19 treatment or *H pylori* infection. An earlier study suggested the role of SRC in the activation of STAT3 in cancer.[54] Inhibition of SRC not only reduced the level of STAT3 but also diminished FGFR4 levels. Based on these findings, we suggest the presence of a novel positive feedforward loop between STAT3 and FGFR4 where SRC serves as a link between FGFR4 and STAT3.

H pylori infection creates a cellular stress condition that can induce cell death in the absence of adaptive survival properties.[55] FGFR4 overexpression can promote cancer cell proliferation, invasion, and metastasis.[56,57] We found that induction of FGFR4 by *H pylori* promoted cell survival and abrogated infection-induced apoptosis. Therefore, the acquired activation of the FGFR4-STAT3 axis can serve as a survival mechanism to promote tumorigenic cells in response to *H pylori* infection. Our findings are in accord with previous studies where FGFR4 silencing mediated a decrease in proliferation with increased apoptosis in ovarian cancer cells.[58] This acquired adaptive survival property in response to *H pylori* may provide cancer cells a resistance phenotype against therapy. Although we did not investigate this function, our analysis of TCGA data demonstrated an association between high levels of FGFR4 and reduced patient survival.

Conclusion

This study demonstrated the role of STAT3 in the activation of FGFR4 signaling and illustrated a positive feedforward loop between STAT3 and FGFR4, where SRC is a critical intermediate. FGFR4 overexpression is relevant to the biology of gastric cancer and calls for

future studies testing FGFR4 inhibitors as a novel therapeutic approach for improving the clinical outcome in gastric cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper:

ChIP	chromatin immunoprecipitation
FBS	fetal bovine serum
FGF19	fibroblast growth factor 19
FGFR	fibroblast growth factor receptor
FGFR4	fibroblast growth factor receptor 4
GSEA	gene set enrichment analysis
H pylori	*Helicobacter pylori*
IL	interleukin
KO	knock-out
mRNA	messenger RNA
p	phosphorylated
PBS	phosphate-buffered saline
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
siRNA	small interfering RNA
SRC	steroid receptor coactivator
STAT3	Signal transducer and activator of transcription 3
TCGA	The Cancer Genome Atlas

TFF1	trefoil factor 1
WT	wild-type

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Public data sets and in vitro and in vivo models were used to investigate the role of *Helicobacter pylori* infection in mediating fibroblast growth factor receptor 4 (FGFR4) oncogenic signaling in gastric carcinogenesis.

NEW FINDINGS

Integrated analysis identified *FGFR4* as the main upregulated FGFR family member. A novel tumorigenic feedforward signaling loop was characterized linking infection, inflammation (signal transducer and activator of transcription 3), and *FGFR4* activation.

LIMITATIONS

This study did not directly address the potential therapeutic significance of FGFR4 inhibitors.

IMPACT

Our findings provide a proof of concept for developing and optimizing therapeutic strategies that test FGFR4 inhibitors as single agents or in combination with chemotherapeutics.

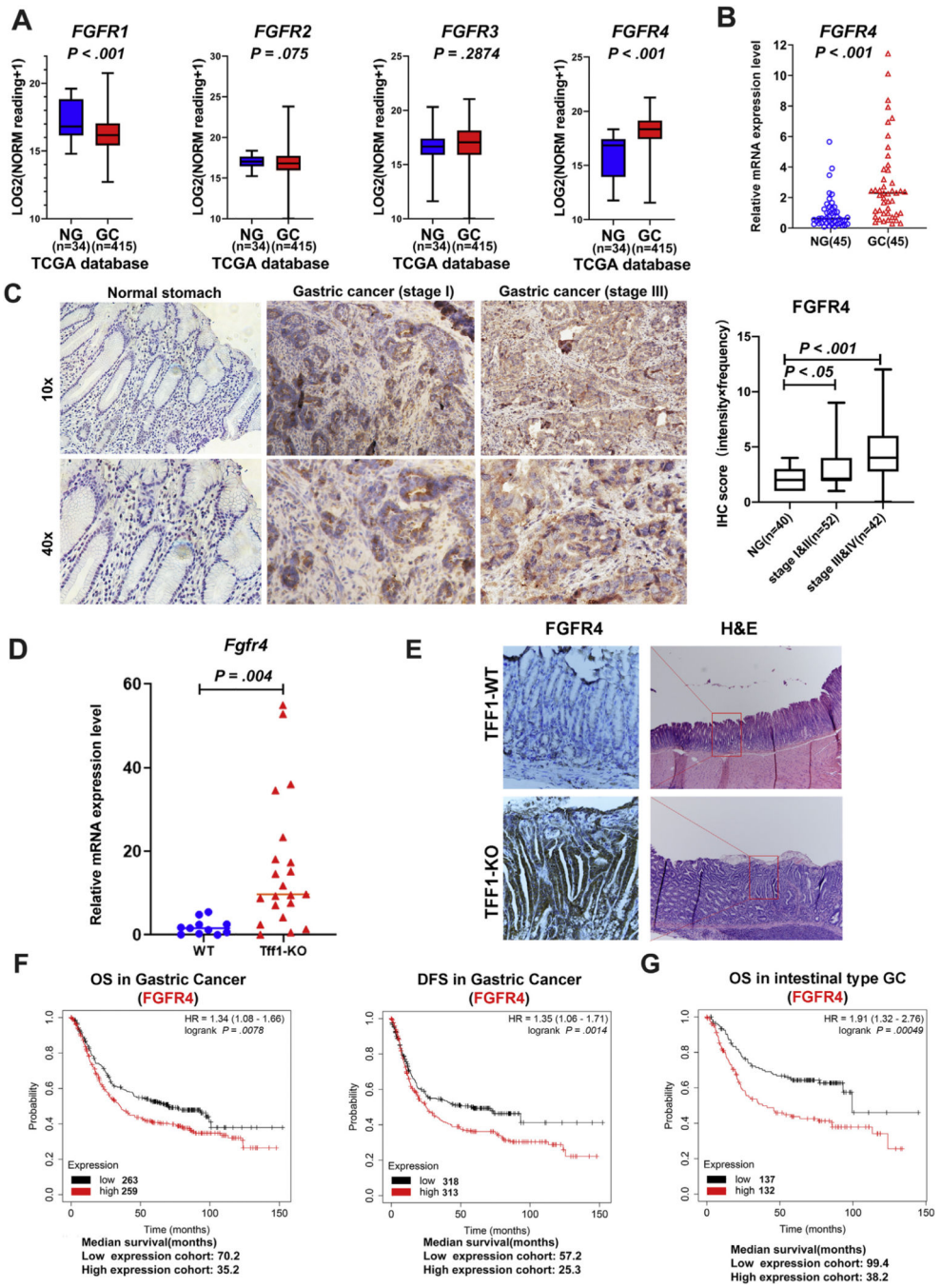

Figure 1.

FGFR4 is upregulated in gastric cancer tissues. (*A*) TCGA database analysis of *FGFR* family members' mRNA expression. *FGFR4* was the most significantly up-regulated *FGFR* in gastric cancer (GC) compared with normal gastric (NG) human tissue. (*B*) qRT-PCR analysis of *FGFR4* mRNA in paired NG and GC human tissues (n = 45). (*C*) Immunohistochemistry (IHC) analysis shows the expression of FGFR4 in normal and human gastric cancer tissue samples (original magnification ×10 is shown in the *upper panels*; insets at original magnification ×40 are shown in the *lower panels*). Quantification

scores are shown on the *right panel*. (*D*) qRT-PCR data demonstrate the expression of *Fgfr4* mRNA in WT (n = 11) and TFF1-KO (n = 22) mouse tissues. (*E*) Immunohistochemistry analysis shows the expression of FGFR4 in neoplastic stomach tissues of the TFF1-KO mice compared with normal (TFF1-WT) (original magnification ×40). H&E staining of the corresponding tissues is shown on the *right panels* (original magnification ×10). (*F*) Kaplan-Meier survival curve shows poor overall survival (OS) and disease-free survival (DFS) in patients with gastric cancer with high expression of *FGFR4*. (*G*) Kaplan-Meier survival curve demonstrates an association between high expression of *FGFR4* and poor overall survival in patients with intestinal-type gastric cancer. HR, hazard ratio. Data are expressed as the means ± standard deviations. Box and whisker plot: The *horizontal line* in the middle of each box indicates the median; the *top and bottom borders* of the box mark the 75th and 25th percentiles, respectively, and the *whiskers* mark the minimum and maximum of all the data. $P < .05$, $P < .01$, and $P < .001$ are considered significant.

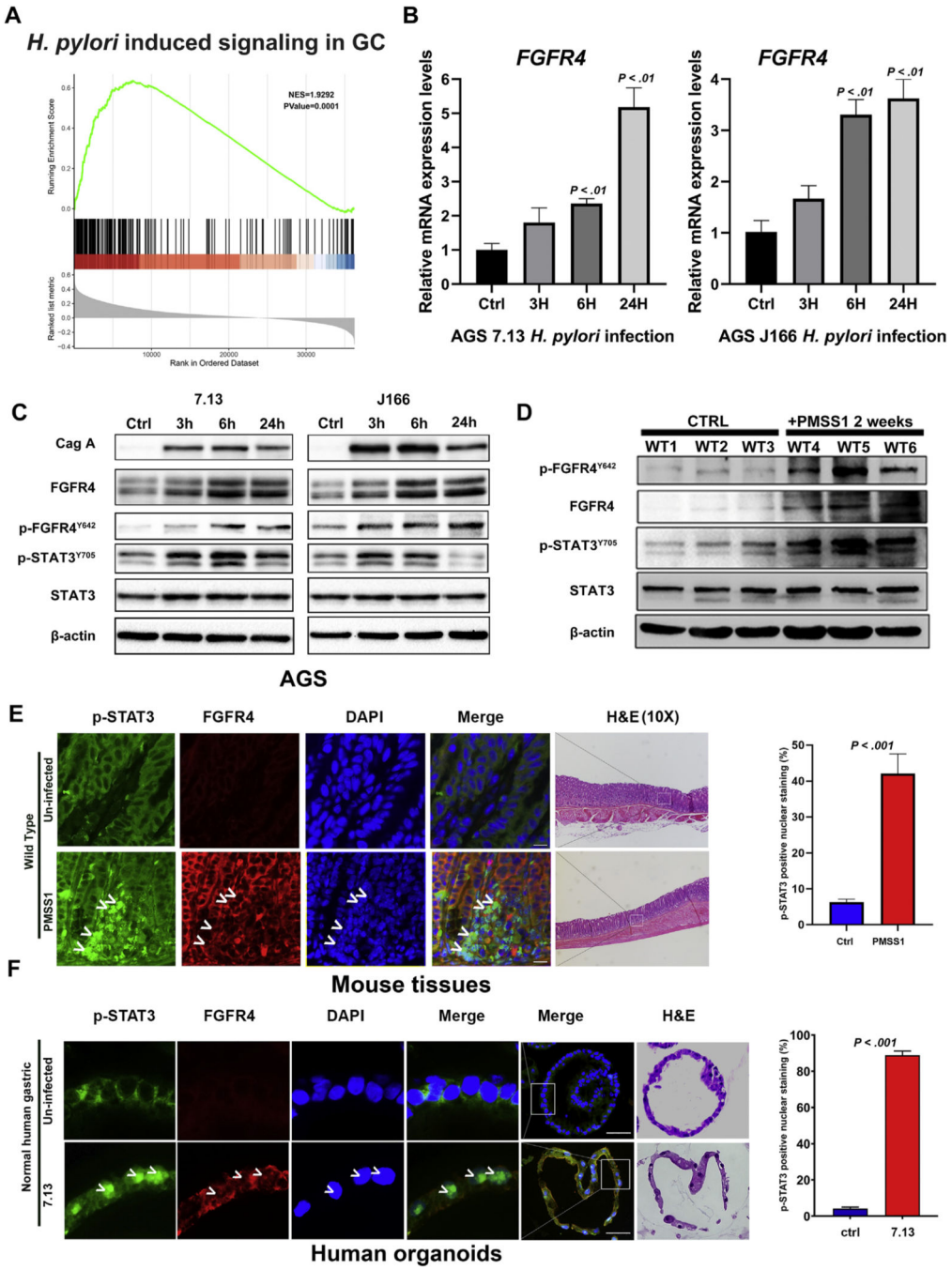

Figure 2.

H pylori infection induces FGFR4 expression and STAT3 activation. (*A*) GSEA based on gene expression analysis of gastric cancers (GCs) in the TCGA database indicated that *FGFR4* has a significant correlation with *H pylori*-induced signaling in gastric cancer. NES, normalized enrichment score. (*B*) The qRT-PCR analysis demonstrates progressive induction of *FGFR4* mRNA expression at different times after *H pylori* infection in AGS cells. Ctrl, control. (*C*) Western blot analysis demonstrates total and p-FGFR4 and p-STAT3 at 3, 6, and 24 hours after *H pylori* infection in AGS cells. Cag A, cytotoxin-associated gene A.

(*D*) Western blot analysis shows total and p-FGFR4 and p-STAT3 in gastric tissues of WT mice infected with PMSS1 *H pylori* for 2 weeks compared with noninfected mice. (*E*) Immunofluorescence data indicate strong expression of FGFR4 and nuclear STAT3 staining in WT gastric mouse tissues infected with PMSS1 *H pylori* (*lower panels*), and weak to absent staining of FGFR4 and p-STAT3 was detected in noninfected mice (*upper panels*) (*scale bar* of 10 μm is shown in the merge image). H&E staining and bright-field images of representative organoids are shown on the *right side* of the panels. DAPI, 4′,6-diamidino-2-phenylindole. Quantification of positive nuclear p-STAT3 staining was performed in at least 200 cells from 3 different fields. Data are presented as a percentage ± standard error of the mean (*right panel*). (*F*) Immunofluorescence data of FGFR4 and nuclear STAT3 staining in uninfected and *H pylori* 7.13–infected organoids derived from normal human gastric tissues. *H pylori*–infected organoids showed strong nuclear p-STAT3 and *FGFR4* staining (*lower panels*) compared with noninfected organoids (*upper panels*). Quantification of positive nuclear STAT3 cells was performed in at least 100 counted cells from 4 organoids (*scale bar* of 50 μm is shown in the merge image). Arrowheads indicate cells with nuclear localization of STAT3. Data are presented as a percentage ± standard error of the mean (*right panel*). H&E staining and bright-field images of representative organoids are shown on the *right side* of the panels. Data are expressed as the means ± standard deviations. $P < .05$, $P < .01$, and $P < .001$ are considered significant.

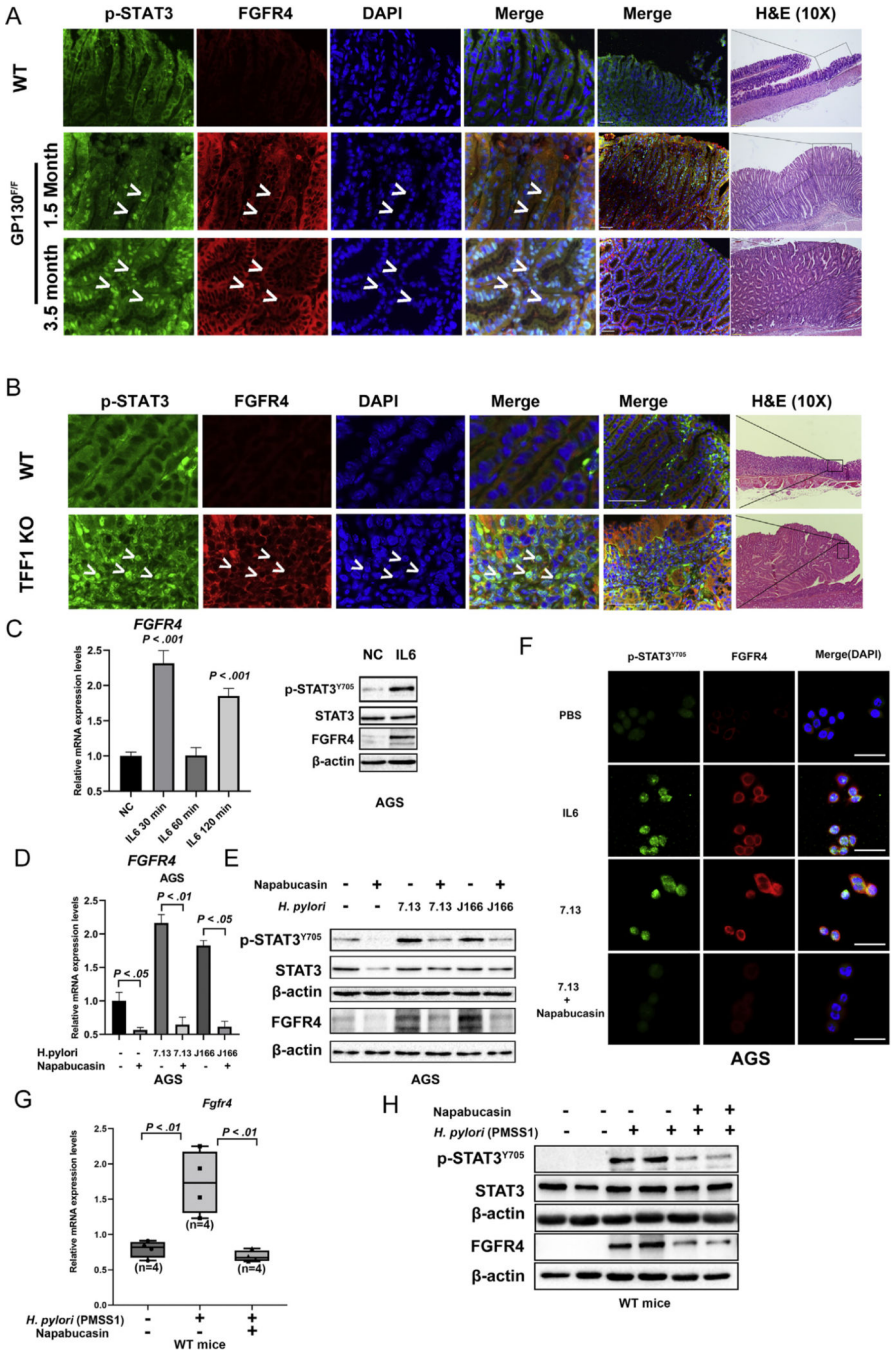

Figure 3.

FGFR4 expression coincides with STAT3 activation in vivo and in vitro. (*A*) Immunofluorescence staining demonstrates an increase in FGFR4 and p-STAT3 (nuclear staining) in gastric tissues from the GP130$^{F/F}$ mouse model (1.5 and 3.5 months old) compared with WT (*scale bar* of 50 μm is shown is the merge image). DAPI, 4′,6-diamidino-2-phenylindole. (*B*) Immunofluorescence staining demonstrates an increase in FGFR4 and p-STAT3 (nuclear staining) in neoplastic gastric tissues from the TFF1-KO mice compared with normal stomach (*scale bar* of 50 μm is shown is the merge image).

Arrowheads indicate cells with nuclear localization of STAT3. (*C*) qRT-PCR data (*left panel*) show an increase of *FGFR4* after IL6 treatment (100 ng/mL) at 30, 60, and 120 minutes in AGS cells. Western blot analysis (*right panel*) confirms the increase in p-FGFR4 and p-STAT3 after IL6 treatment. NC, negative control. (*D*) qRT-PCR data indicate an increase of *FGFR4* expression after *H pylori* infection (J166 or 7.13 strains) in AGS cells. This effect is reversed after STAT3 inhibitor treatment (0.4 μmol/L napabucasin, overnight). (*E*) Western blot data confirm the induction of p-FGFR4 and p-STAT3 after *H pylori* infection in AGS cells, an effect that is abrogated by napabucasin treatment (STAT3 inhibitor). (*F*) Immunofluorescence indicates that both IL6 treatment and *H pylori* infection induce expression of FGFR4 and nuclear p-STAT3 staining in AGS, whereas napabucasin inhibits the expression of both FGFR4 and p-STAT3 (*scale bar* of 50 μm is shown is the merge image). Data are expressed as the means ± standard deviations. $P < .05$, $P < .01$ and $P < .001$ are considered significant. (*G*) qRT-PCR data show an increase of *Fgfr4* mRNA expression in WT mice stomach after *H pylori* (PMSS1) infection. This increase was abolished after treatment with napabucasin (40 mg/kg). Box and whisker plot: The *horizontal line* in the middle of each box indicates the median; the *top and bottom borders* of the box mark the 75th and 25th percentiles, respectively, and the *whiskers* mark the minimum and maximum of all the data. (*H*) Western blot analysis confirms the expression of FGFR4 and p-STAT3 after *H pylori* PMSS1 infection in mice. This effect was abrogated by napabucasin treatment.

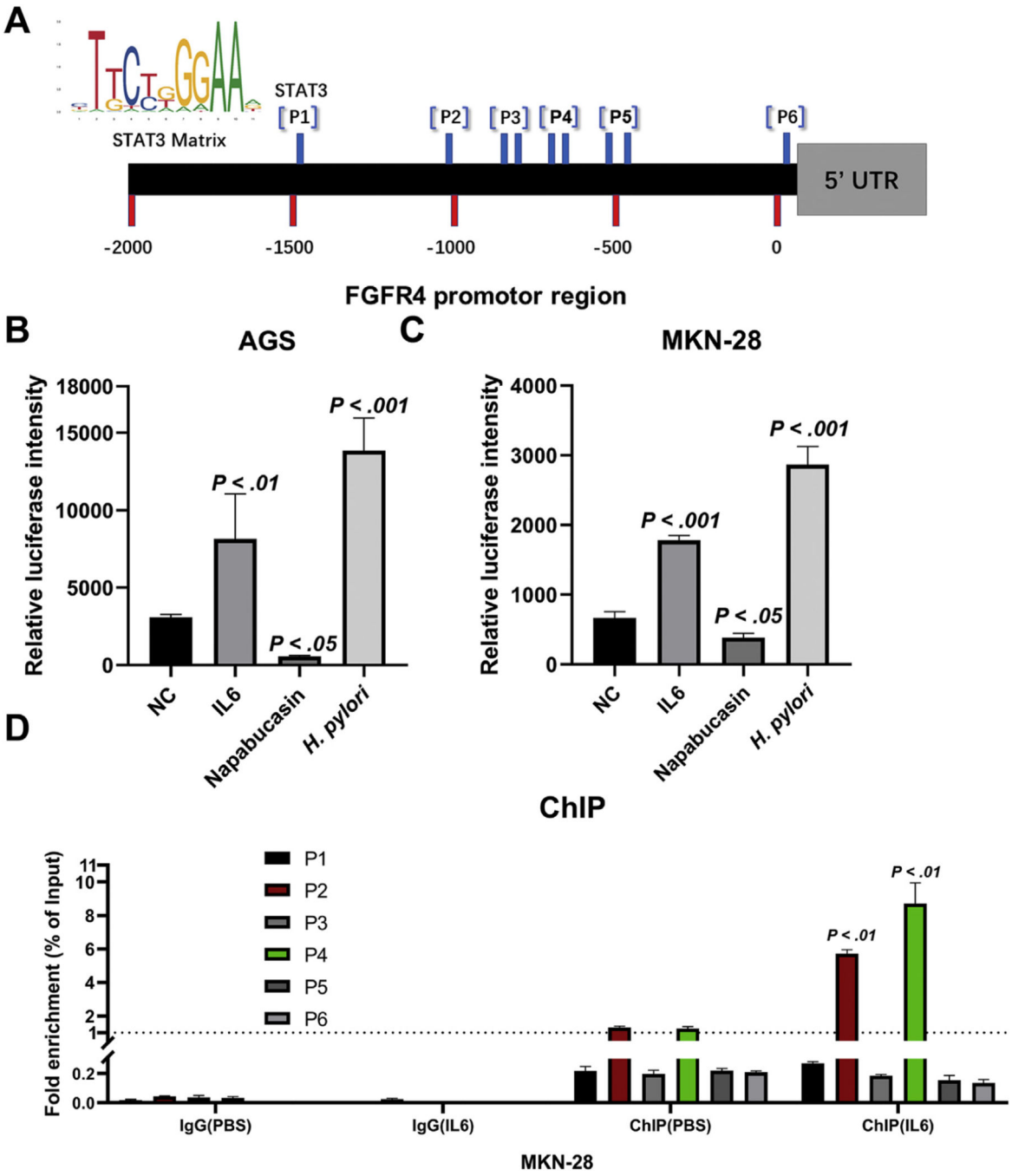

Figure 4.

FGFR4 expression is induced via direct binding of STAT3 on the FGFR4 promoter. (*A*) A schematic diagram shows the location of STAT3 putative binding regions on the *FGFR4* promoter. ChIP assay primers were designed to cover regions P1 to P6. UTR, untranslated region. FGFR4 luciferase promoter-reporter assay demonstrates an increase in the luciferase activity in (*B*) AGS and (*C*) MKN-28 cells after *H pylori* infection (8 hours) or IL6 treatment (100 ng/mL, 6 hours). NC, negative control. Inhibition of STAT3 by napabucasin abrogated the luciferase induction under these conditions. (*D*) ChIP assay

showing amplification of DNA fragments quantified by qRT-PCR and demonstrating the binding of STAT3 on the FGFR4 promotor in both P2 and P4 regions. Data are expressed as the means ± standard deviations. $P < .05$, $P < .01$ and $P < .001$ are considered significant.

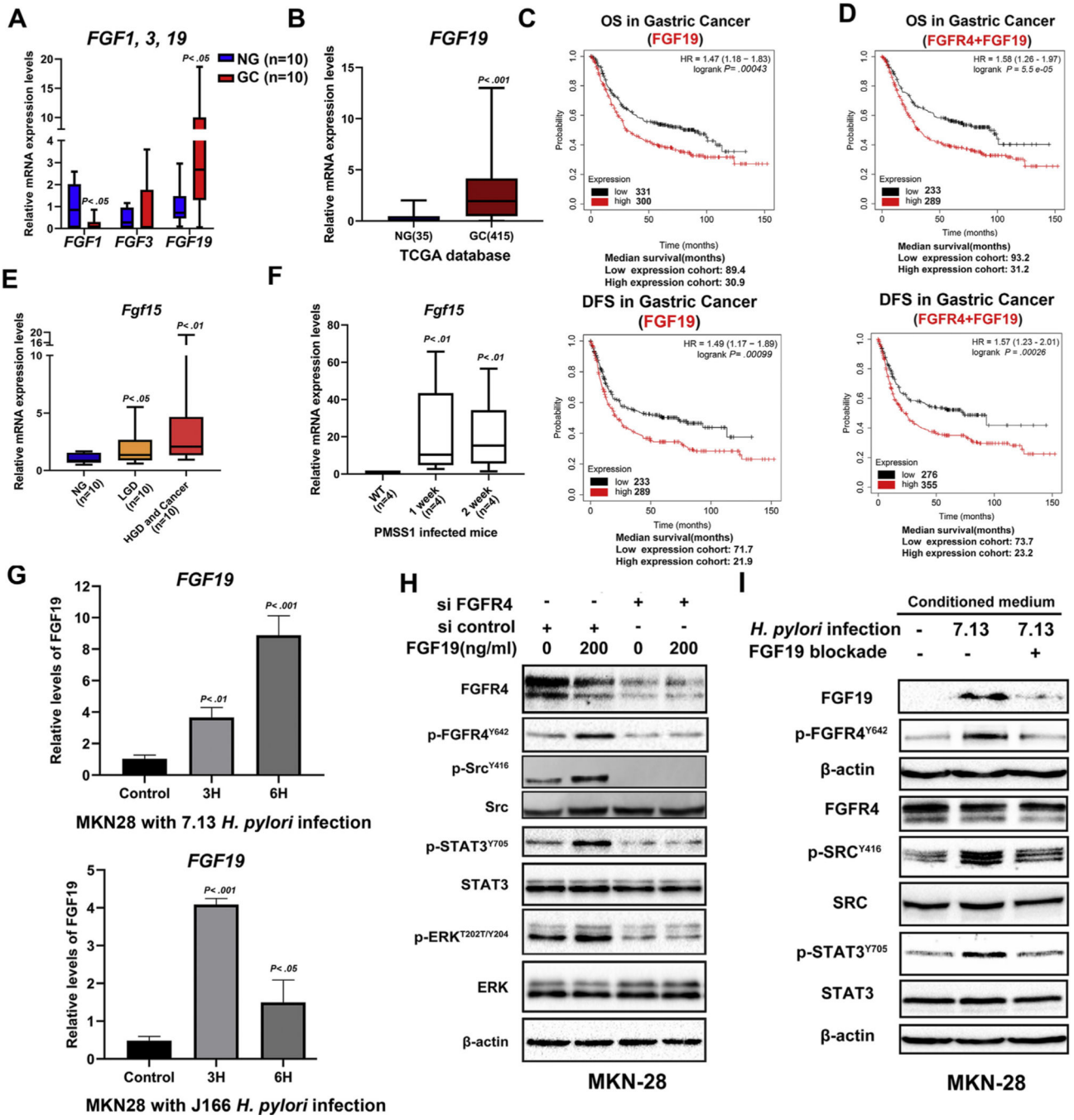

Figure 5.

The FGF19-FGFR4 axis promotes a feedforward activation loop of STAT3 via SRC. (*A*) qRT-PCR analysis shows *FGF1*, *FGF3*, and *FGF19* expressions in human gastric cancer (GC) compared with normal glandular (NG) stomach tissues. (*B*) TCGA database analysis indicated that *FGF19* mRNA was significantly upregulated in GC compared with NG tissues. Kaplan-Meier survival curve indicates that patients with GC with high expression of (*C*) *FGF19* and (*D*) *FGFR4*+*FGF19* have poor overall survival (OS) and poor disease-free survival (DFS) compared with patients with GC with low expression. HR, hazard

ratio. (*E*) qRT-PCR analysis demonstrates upregulation of *FGF19* at low-grade dysplasia (LGD) and high-grade dysplasia (HGD)/cancer tissue samples from the TFF1-KO mice compared with normal gastric tissues. (*F*) qRT-PCR analysis demonstrates upregulation of *Fgf15* mRNA expression after 1 and 2 weeks of PMSS1 *H pylori* infection in WT mice stomachs. Box and whisker plot: The *horizontal line* in the middle of each *box* indicates the median; the *top and bottom borders* of the box mark the 75th and 25th percentiles, respectively, and the *whiskers* mark the minimum and maximum of all the data. (*G*) qRT-PCR analysis demonstrates *FGF19* mRNA expression is induced at different times after *H pylori* infection in MKN28 cell lines. Data are expressed as the means ± standard deviations. $P < .05$, $P < .01$ and $P < .001$ are considered significant. (*H*) Western blot analysis is shown in MKN28 cells transfected with control or FGFR4 siRNA. After stimulation with FGF19 (200 ng/mL), MKN28 cells demonstrate a remarkable increase of p-FGFR4, p-STAT3, p-SRC, and p-extracellular signal–regulated kinase (ERK). In contrast, all of these proteins are significantly reduced after FGFR4 siRNA knockdown, with or without FGF19 stimulation. (*I*) Western blot analysis indicates that FGF19 neutralizing antibody blocks the *H pylori*-mediated increase in p-FGFR4, pSTAT3, p-SRC, and p-ERK compared with conditioned media without neutralizing antibody. The secretion of FGF19 is confirmed in the conditioned media of MKN28 cells.

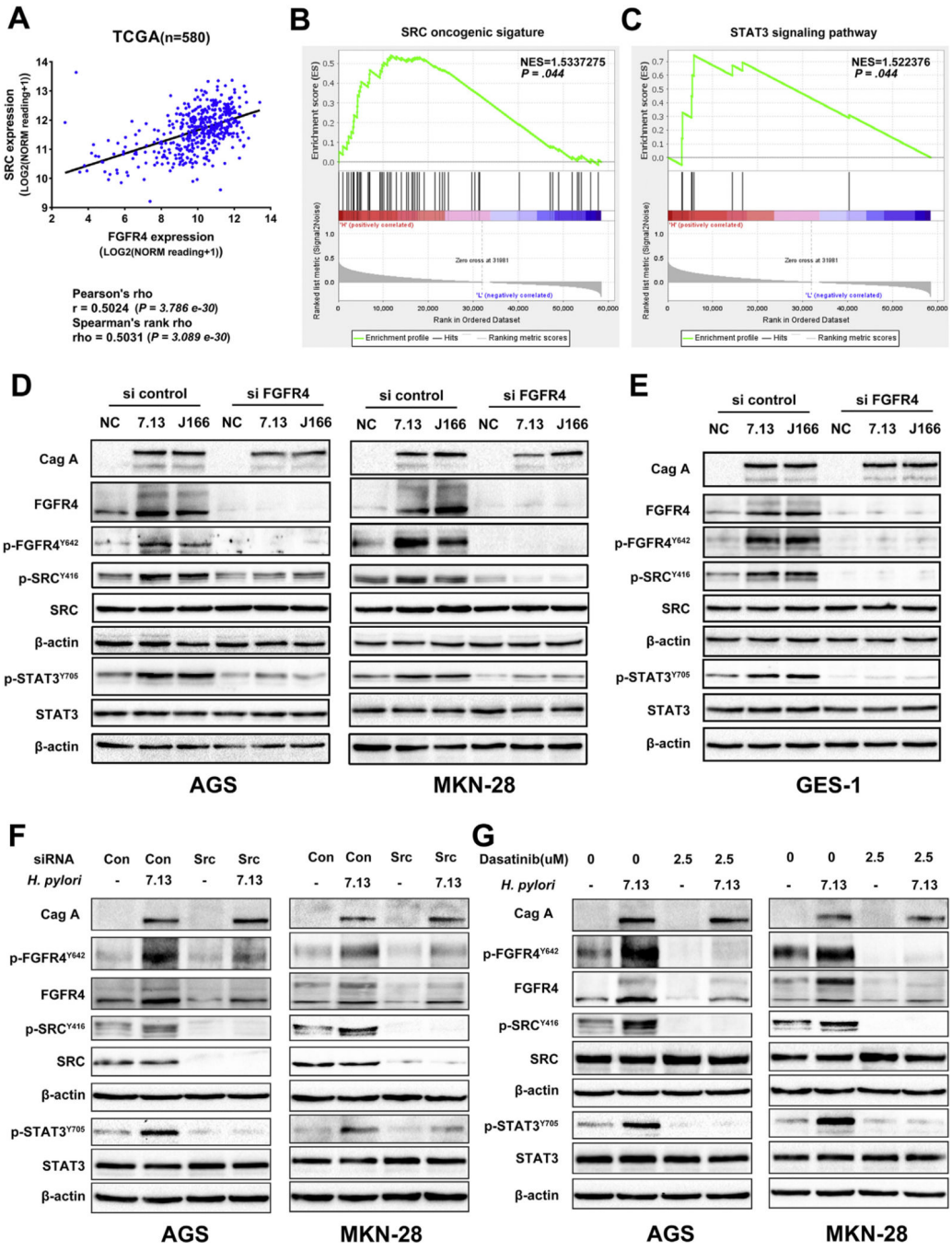

Figure 6.

The FGFR4-SRC axis is crucial for the *H pylori*-induced activation of STAT3 signaling. (*A*) Pearson's correlation of *FGFR4* and *SRC* mRNA levels using the TCGA database. GSEA analysis indicates a positive correlation between *FGFR4* and the (*B*) SRC and (*C*) STAT3 signaling pathways in gastric cancer. NES, normalized enrichment score. (*D* and *E*) Western blot analysis shows an increase in FGFR4, SRC, and STAT3 phosphoproteins after *H pylori* infection. This increase is abolished after the knockdown of FGFR4 using siRNA in AGS, MKN28, and GES-1 cells. Cag A, cytotoxin-associated gene A; NC, negative

control. (*F*) Western blot analysis indicates that SRC knockdown after *H pylori* infection decreases the expression p-FGFR4, p-SRC, and p-STAT3 compared with control siRNA in AGS and MKN28 cells. (*G*) Western blot analysis, after inhibition of SRC using dasatinib, demonstrates a decrease in the expression of p-FGFR4, p-SRC, and p-STAT3 after *H pylori* infection compared with control siRNA in AGS and MKN28 cells.

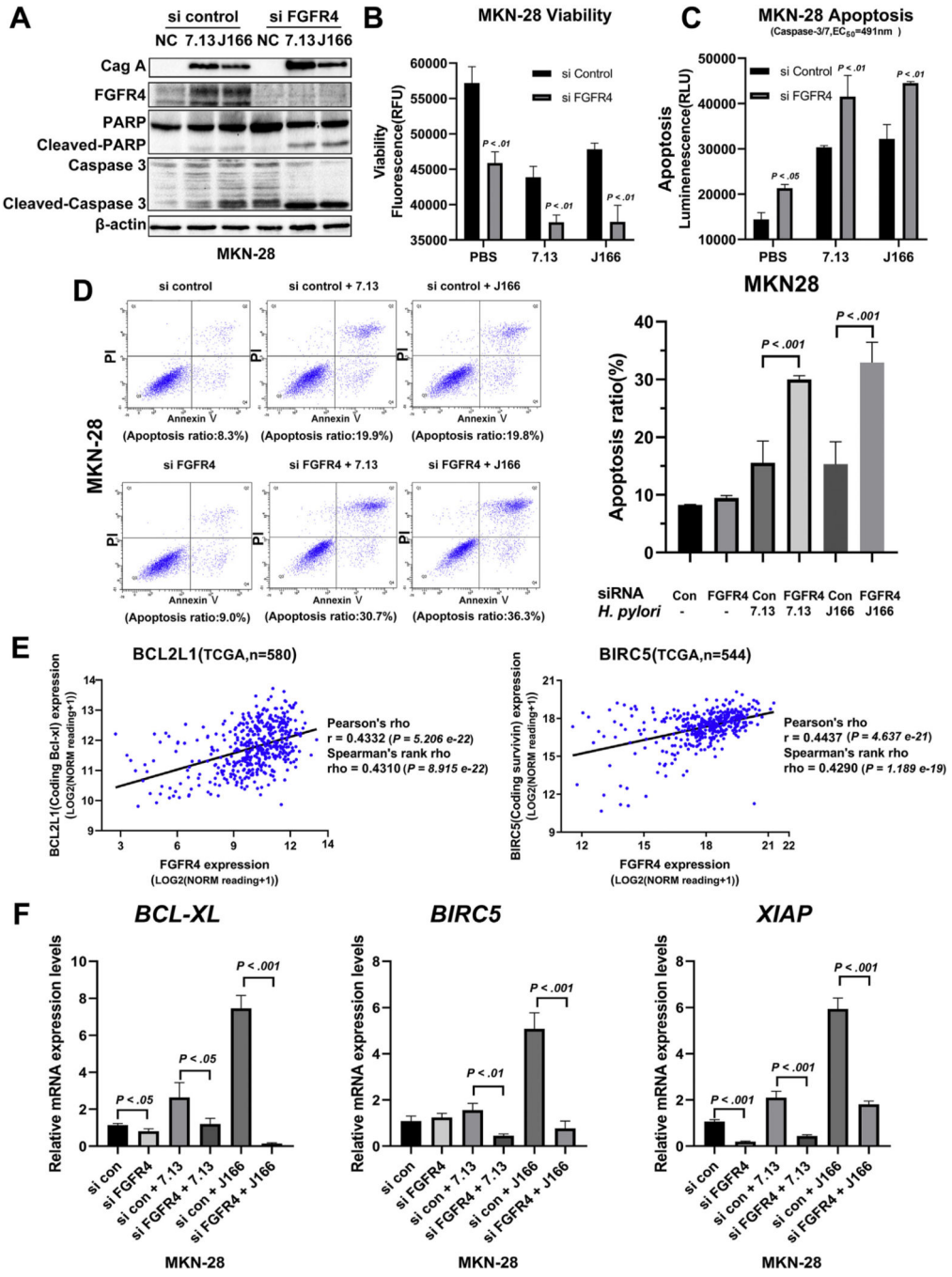

Figure 7.

FGFR4 protects gastric cancer cells from apoptosis during *H pylori* infection. (*A*) Western blot analysis shows an increase of cleaved poly (adenosine-5′-diphosphate–ribose) polymerase (PARP) and cleaved-caspase 3, apoptotic markers, after knockdown of FGFR4 in *H pylori*-infected MKN28 cells. Cag A, cytotoxin-associated gene A; NC, negative control. (*B* and *C*) ApoTox-Glo assay data show decreased cell viability and increased caspase 3/7 activity after knockdown of FGFR4 in *H pylori*-infected MKN28 cells. ED_{50}, 50% effective dose. RFU, relative fluorescence units; RLU, relative luminescence unit.

(*D*) Annexin V/propidium iodide (PI) staining flow cytometry analysis demonstrates a significant increase in apoptotic cells after knockdown of FGFR4 in *H pylori*-infected MKN28 cells compared with control cells. The apoptosis quantification ratio is presented on the *right panel*. (*E*) Pearson's correlation test is shown for *FGFR4* and *BCL2L1* mRNAs (left panel) and *FGFR4* and *BIRC5* mRNAs, using TCGA data. (*F*) A qRT-PCR analysis is shown for prosurvival genes (*BCL-XL*, *BIRC5*, and *XIAP*) in control and FGFR4 siRNA conditions, with or without infection with J166 or 7.13 *H pylori* strains for 24 hours in MKN28 cells. All genes displayed an increase in mRNA expression levels in response to *H pylori* infection. This increase is attenuated after the knockdown of FGFR4. Data are expressed as the means ± standard deviations. $P < .05$, $P < .01$ and $P < .001$ are considered significant.