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# Estrogen-related receptor-gamma influences *Helicobacter pylori* infection by regulating TFF1 in gastric cancer

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ABSTRACT

*Helicobacter pylori* infection is a crucial factor in the development of gastric cancer (GC). Molecular therapeutic targets and mechanisms contributing to *H. pylori* infection-associated GC induction are poorly understood and this study aimed to fill that research gap.

We found that the nuclear receptor estrogen-related receptor gamma (ESRRG) is a candidate factor influencing *H. pylori* infection-driven GC. ESRRG suppressed *H. pylori* infection and cell growth induced by *H. pylori* infection in GC cells and organoid models In addition, *H. pylori* infection downregulates ESRRG expression. Gene expression profiling revealed that trefoil factor 1 (*TFF1*), a well-known tumor suppressor in GC, is a downstream target of ESRRG. Mechanistically, ESRRG directly binds to the *TFF1* promoter and induces TFF1 gene expression. Furthermore, TFF1 activation by ESRRG was inhibited by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)/p65, which is induced by inflammation, such as by *H. pylori* infection.

Our current study provides new molecular insights into how ESRRG regulates *H. pylori* infection, contributing to GC development. We suggest that modulation of ESRRG-suppressing *H. pylori* infection could be a therapeutic target for the treatment of GC patients.



## 1. Introduction

Gastric cancer (GC) is the most common cancer globally and predominantly in Eastern Asia [1]. While surgical resection is the standard regimen for early-stage GC, use of standard treatment in the advanced and late stages is very limited [2]. Several molecular therapeutic options using monoclonal antibodies, such as trastuzumab and bevacizumab, have been widely used for GC patients [3]. Recently developed immune therapies modulating T-cell activation have emerged as a new therapeutic option [4]. However, immunotherapy has minimal success rate, demonstrating that effective new molecular targets for treatment are urgently required [4–6].

Since the detailed molecular mechanism of GC development is poorly understood, the development of therapeutic targets is delayed, suggesting that a biological understanding of GC is crucial for treatment regimens [7,8]. Of the many factors contributing to GC, *Helicobacter pylori* infection, classified as a class I carcinogen by the WHO, is a causative agent of GC [9]. *Helicobacter pylori* infection leads to chronic inflammation, which eventually promotes GC [10]. However, the molecular mechanisms governing the relationship between *H. pylori* infection and GC development are not clearly understood.

The imbalance between tumor suppressors and oncogenes influences GC development. For example, conventional tumor suppressors TP53 and PTEN play a crucial role in tumorigenesis [8] and RUNX3 [11] and ESRRG [12] act as gastric-specific tumor suppressors due to their restricted expression patterns. Deregulation of tumor suppressors is a critical step in tumorigenesis, although the molecular mechanisms of tumor suppression vary. Thus, modulation of tumor-suppressive transcription factors is well recognized as an effective therapeutic strategy for cancer [12].

*Helicobacter pylori* infection is a major contributing factor to GC [13]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a well-known transcriptional regulator of inflammation, is induced by *H. pylori* and is involved in GC development [14]. However, the relationship between tumor suppressors and *H. pylori* infection is not well established in GC. Previously, we identified that ESRRG suppresses the Wnt pathway as a new tumor suppressor in

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GC [12]. Since ESRRG inhibits GC development by suppressing the Wnt pathway, which positively influences *H. pylori* infection, we can assume that ESRRG can modulate *H. pylori* infection.

In the current study, we identified that ESRRG protects gastric cells from *H. pylori* infection and inhibits GC cell growth by regulating TFF1 and NF-κB. ESRRG is positively correlated with TFF1 and negatively correlated with NF-κB. Our study provides new molecular insights into the correlation between ESRRG and *H. pylori* infection, and suggests that ESRRG can be a therapeutic target for the treatment of GC.

## 2. Materials and methods

### 2.1. Gene expression data analysis

Gene expression data from the NCBI Gene Expression Omnibus (GEO) database are publicly available. All data were downloaded and processed using Biometric Research Branch (BRB) array tools and used for further analysis.

### 2.2. Cell lines and reagents

GC cell lines were purchased from the American Type Culture Collection. Cells were grown in Dulbecco's modified essential medium or RPMI1640 supplemented with 10% fetal bovine serum. The cells were incubated at 37 °C in a humidified incubator with 5% CO2. DY131 (#2266; TOCRIS, Bristol, UK) and GSK5182 (#AOB1629; Aobious, Gloucester. MA, USA) were purchased.

### 2.3. Cell proliferation assay

Stably or transiently transfected cells were used for cell growth assays. The proliferation assay was performed following the manufacturer's instructions (CCK8-Kit; CK04-20, Dojindo, Rockville, MD, USA).

### 2.4. Colony-forming assay

Cells were infected with lentivirus or treated with the indicated compounds for a designated time. Cells (500 μL) were seeded into 6-well plates. Two weeks later, the cells were fixed with 3.7% paraformaldehyde for 5 min and stained with 0.05% crystal violet for 15 min. Colonies containing more than 50 cells were counted.

### 2.5. Reporter assay

pGL3-*TFF1* and pCDNA3-ESRRG cDNAs have been described previously [12]. For luciferase-based reporter assays, cells were transfected with the indicated reporter genes and plasmids (pGL3-*TFF1* and ESRRG) using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. After 48 h, the cells were harvested to measure luciferase activity.

### 2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed according to the protocol from Thermo Scientific, Pierce™ Magnetic ChIP Kit (#26157) with minor modifications [12,15]. The antibodies used were specific for ESRRG (PP–H6812-00; R&D Systems, Minneapolis, MN) and standard mouse immunoglobulin G (SC-2027; Santa Cruz Biotechnology, Dallas, TX).

### 2.7. Western blot

Western blot analysis was performed as described previously. The antibodies used were specific for ESRRG (#H6812; R&D Systems), TFF1 (#9702; Cell Signaling Technology [CST], Danvers, MA), β–actin (A5316; Sigma-Aldrich), and α–tubulin (#3873; CST). Antibodies were diluted with bovine serum albumin (BSA) and used at a 1:1000 ratio, after blocking the membrane with BSA.

### 2.8. qRT-PCR

qRT-PCR was performed according to the protocol using the mirVana RNA isolation kit (Ambion, Austin, TX) according to the manufacturer's instructions as described previously [12,15].

### 2.9. Lentiviral transduction

Lentivirus was made and transduced to the cell as described previously [12,15].

### 2.10. Helicobacter pylori infection

For AGS cells or organoid infections, *H. pylori* (ATCC43504, type strain 11637, *cagA* $^{+}$ *vacA*$^{+}$) was grown under microaerophilic conditions for 48 h on Brucella broth agar plates containing 7% sheep blood and added to AGS cell monolayers for 4 h at a multiplicity of infection (MOI) of 50.

### 2.11. Organoid culture

The organoid culture was based on a previous report, with minor modifications [12,16]. Gastric fundus organoids were derived from surgical samples from patients with gastric cancer at Yensei University Severance Hospital (IRB No. 4-2015-0877).

### 2.12. Confocal laser scanning microscopy and live-cell imaging

Fluorescence microscopy was performed as described previously [12]. LysoTracker-Red DND-99 (ThermoFisher, Waltham, MA) was detected by excitation at 561 nm for AGS cells and mouse organoids. For dual-color observation, GFP and LysoTracker-Red DND-99 were detected by excitation at 488 nm. Microscopy images were processed and analyzed using ZEN2012 software installed on LSM780.

## 3. Results

### 3.1. ESRRG has protective roles against H. pylori infection in gastric cells

Previously, we found that ESRRG functions as a tumor suppressor by antagonizing the Wnt pathway in GC, and ESRRG expression was found to be predominant in normal gastric tissues [12]. In addition, gene expression profiling data revealed that *H. pylori* infection suppresses ESRRG expression [17]. Since ESRRG antagonizes tumor growth and is influenced by *H. pylori* infection, we hypothesized that the tumor-suppressive activity of ESRRG is correlated with *H. pylori* infection.

To investigate how *H. pylori* infection affects ESRRG expression, after *H. pylori* infection of AGS cells, the cells were stained with Lyso Tracker Red, which stains acidic cells, a good indicator of *H. pylori* infection (Fig. 1a). ESRRG expression was decreased in *H. pylori*-infected AGS cells according to previously reported data (Fig. 1a) [17]. Since *H. pylori* infection suppressed ESRRG expression, we hypothesized that ESRRG has a protective role against *H. pylori* infection. After AGS cells were infected with lentivirus to overexpress ESRRG, *H. pylori* infection was induced in AGS cells. The results showed that AGS cells were infected with *H. pylori.*

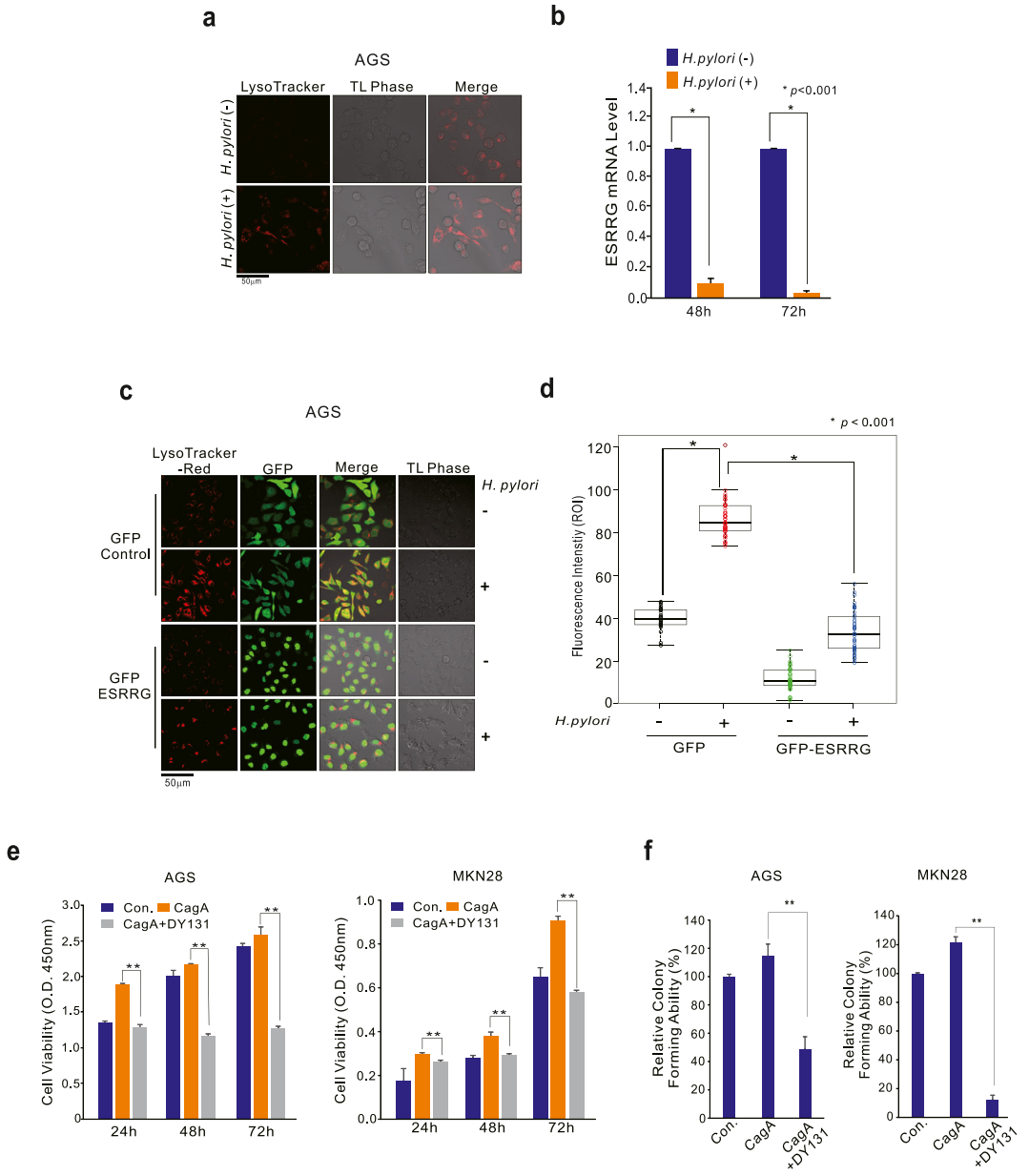


**Fig. 1.** ESRRG modulation in *Helicobacter pylori*-infected cells (a) AGS cells were infected with *H. pylori* and stained with Lysotracker. (b) Infected cells were harvested at the indicated time points and used for qRT-PCR. (c) AGS cells were infected with GFP or GFP-ESRRG lentivirus and selected with puromycin. After lentivirus infection, the cells were infected with *H. pylori* for 5 h, and Lyso Tracker Red DND-99 staining was performed. Scale bar = 50 μM. (d) The cells stained with Lyso Tracker Red DND-99 were quantified by measuring the fluorescence intensity. (e). Indicated gastric cancer cells were transfected with the *CagA* construct and treated with DY131 for indicated times. The cells were then used for CCK8 and colony formation assays. Data represent the mean ± s.d. from three independent replicates (*$p < 0.05$, **$p < 0.01$, *$p < 0.005$ by Student's *t*-test if not indicated).

However, ESRRG-overexpressing AGS cells were less infected than parental AGS cells (Fig. 1c). These results were quantified based on fluorescence intensity (Fig. 1d), demonstrating that ESRRG may have a protective function against *H. pylori* infection.

*Helicobacter pylori* infection contributes to cancer cell growth, and the pathogenic protein cytotoxin-associated gene A (CagA) plays critical roles in inflammation-induced GC [17,18]. To investigate whether ESRRG could influence CagA-induced cancer cell growth, we performed a cell proliferation assay. Instead of ESRRG overexpression, we used the ESRRG activator, DY131, as performed previously [12]. CagA induced GC cell growth, as expected, and DY131 efficiently and significantly inhibited GC cell growth by CagA (Fig. 1e). In addition, the increase in colony formation by CagA was also decreased by DY131 (Fig. 1f). Taken together, our results suggest that ESRRG could function as a protector against *H. pylori* infection to maintain tumor-suppressive activity, and ESRRG deregulation may be necessary for the development of GC.

### 3.2. Evaluation of ESRRG roles during H. pylori infection in in vivo gastric models

We examined the role of ESRRG in *H. pylori* infection using a mouse organoid culture system, which reflects *in vivo* physiology and is widely used for testing gene function and drug efficacy [19]. Since ESRRG expression is high in normal gastric tissues, we inhibited ESRRG activity using an ESRRG inverse agonist GSK5182 [20]. Mouse organoids were treated with GSK5182 before *H. pylori* infection. GSK5182-treated organoids were more susceptible to

*H. pylori* infection (Fig. 2a and b). In addition, ESRRG expression was diminished during *H. pylori* infection, accompanied by CagA protein expression (Fig. 2c). Thus, the results of the organoid experiments were in agreement with the cell culture data. To investigate the efficacy of an ESRRG agonist, we adopted an organoid model from human patient samples. As expected, the ESRRG agonist DY131, which can enhance ESRRG activity-treated human organoids, showed diminished infection by *H. pylori*, suggesting that DY131 has protective potential against *H. pylori* infection (Fig. 2d and e). Taken together, these results suggest that ESRRG plays a vital role in mediating *H. pylori* infection in GC.

### 3.3. Tumor suppressor TFF1 is a downstream target of ESRRG in gastric cancer

ESRRG functions as a transcription factor and modulates diverse downstream target genes to maintain its molecular and physiological functions. Therefore, to investigate downstream targets reflecting ESRRG molecular features, we generated a gene expression profile. After ESRRG was stably overexpressed in GC cells, AGS, and MKN-45, we constructed gene expression profiles using microarray experiments (GSE78050) [12]. Gene expression profiling revealed that 3009 genes were differentially regulated between the control and ESRRG overexpression (OE) groups in two different GC cell lines. A Venn diagram was generated and showed that a substantial number of genes were identified as downstream targets of ESRRG. Differentially expressed genes were visualized depending on the fold ratio (Fig. 3a). Of the 3009 genes, potential oncogenic genes such as *CCND1*, *SKP1*, *JAG1*, *RANBP1*, and *BUB3* were downregulated by ESRRG overexpression, as expected. Since ESRRG harbors tumor-suppressive activity, we selected *TFF1* (Trefoil Factor 1) as ESRRG target. Previous reports suggest that TFF1 is a potent tumor suppressor and is correlated with *H. pylori* infection in GC [21,22]. Thus, we hypothesized that the tumor-suppressive role of *ESRRG* in preventing *H. pylori* infection is through the regulation of TFF1 in GC.

We measured TFF1 protein and mRNA levels after ESRRG overexpression in GC cells to validate the gene expression profile data. ESRRG upregulated TFF1 expression in GC cells (Fig. 3b and c). In addition, the ESRRG agonist DY131 also activated the *TFF1* gene expression (Fig. 3d). Next, we tested whether ESRRG directly regulated *TFF1* gene expression. We performed a reporter assay using the *TFF1* promoter in GC cells. *TFF1* promoter activity was significantly enhanced by ESRRG in a dose-dependent manner (Fig. 3e). We found ESRRG-binding sites in the *TFF1* promoter region (Fig. 3f). Using ChIP assays, we confirmed that ESRRG directly

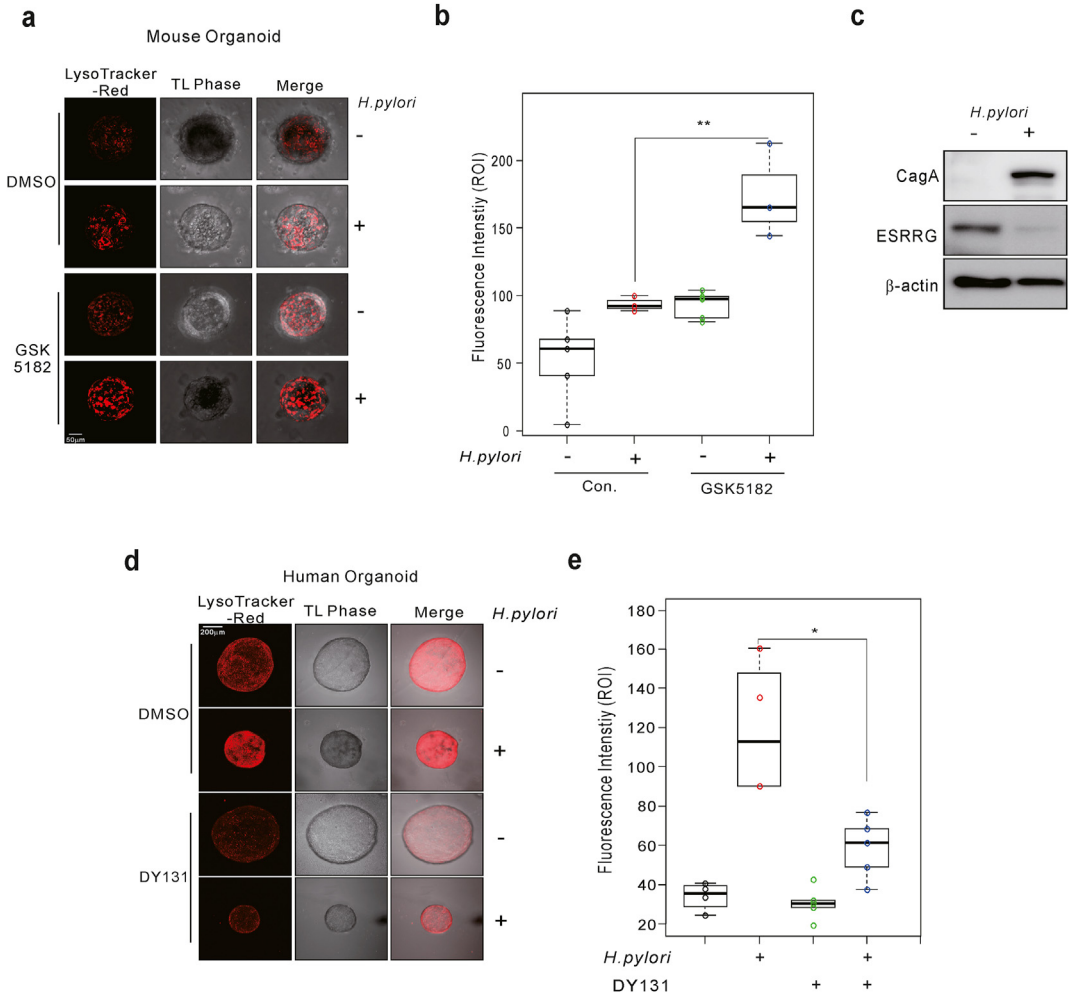


**Fig. 2.** ESRRG expression and *Helicobacter pylori* infection in organoid model. (a–c) Mouse gastric organoids were incubated with GSK5182 and infected with *H. pylori* for 5 h. The organoids were stained with Lyso Tracker Red DND-99 for quantification and used for Western blot with indicated antibodies (c). (d–e) Human gastric organoids were infected with *H. pylori* and stained with Lyso Tracker Red DND-99 for quantification. Data represent the mean ± s.d. from indicated samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

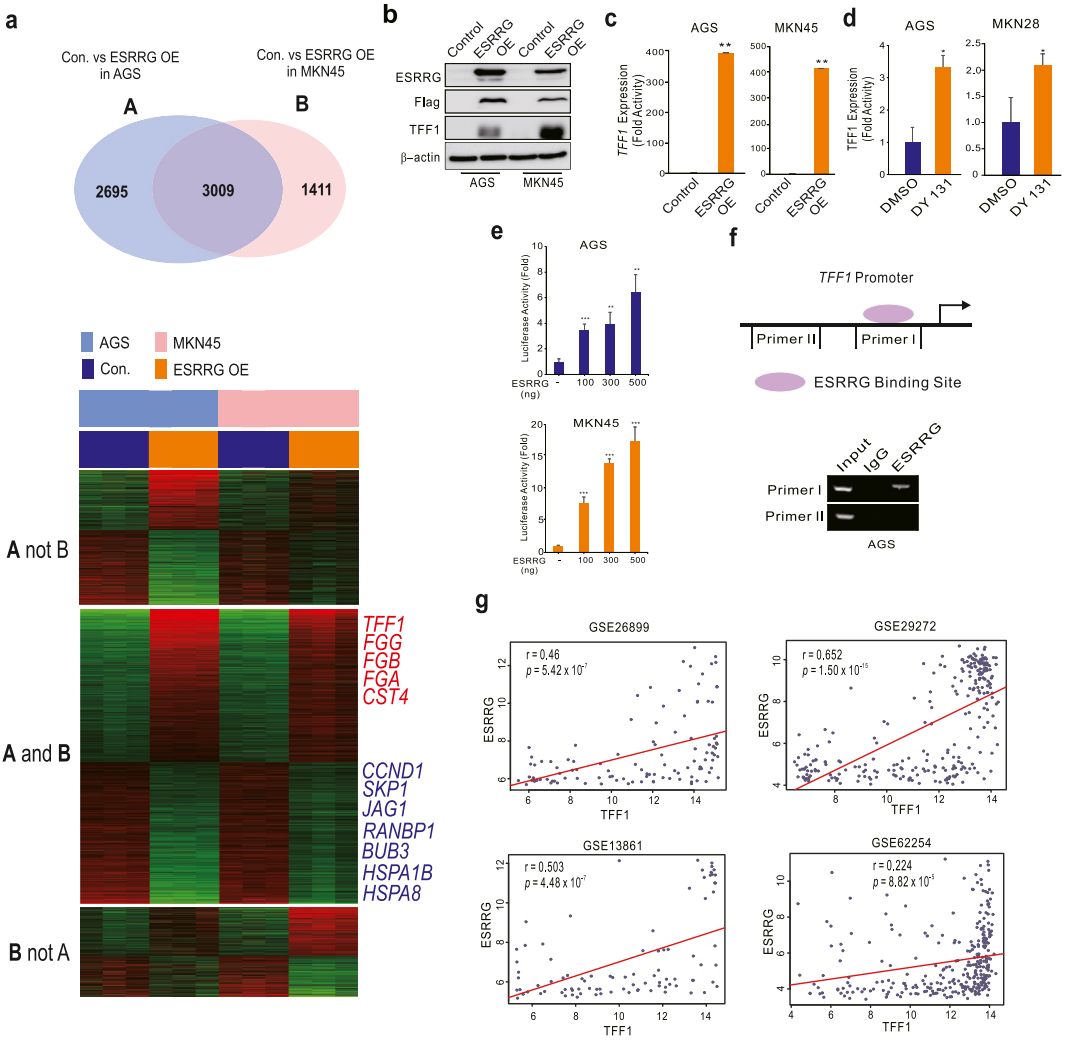


**Fig. 3.** *TFF1* is a direct target of ESRRG in gastric cancer (a) Gene expression signature of ESRRG in Con. or ESRRG lentivirus transduced AGS and MKN45 cells. Genes in the Venn diagram were selected by applying Class-Comparison in BRB-array tool ($p < 0.001$) comparing Con. and ESRRG OE samples. Red and green shows relatively high and low expression levels of genes, respectively (b–d). Western blot (b) and qPCR (c–d) analysis of TFF1 in gastric cancer cells (AGS and MKN45) after cells were infected with Control or ESRRG lentivirus. (d) Indicated cells were treated with DY131 for 72 h and harvested for qRT-PCR analysis. (e) AGS and MKN45 cells were transiently transfected as indicated with the *TFF1* reporter and ESRRG construct. (f) ChIP assays were performed on AGS samples with an ESRRG antibody. Recruitment of ESRRG to the *TFF1* promoter was analyzed using primers specific to the *TFF1* promoter. IgG was used as an internal control. (g) Correlation of ESRRG and TFF1 expression in indicated GC patient cohorts. Scatter plots of ESRRG and TFF1 in gastric cancer cohorts. Data represent the mean ± s.d. for indicated samples (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ by Student's *t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

binds to the *TFF1* promoter region (Fig. 3f). Since ESRRG tightly regulates TFF1 gene expression in GC cells, we investigated whether ESRRG is positively correlated with TFF1 in GC samples. A strong correlation between ESRRG and TFF1 was observed in various GC patient cohorts ($r = 0.46$, $p = 5.42 \times 10^{-7}$ in the GSE26899 cohort; $r = 0.652$, $p = 1.5 \times 10^{-15}$ in the GSE29272 cohort; $r = 0.503$, $p = 4.48 \times 10^{-7}$ in the GSE13861 cohort; $r = 0.224$, $p = 8.82 \times 10^{-5}$ in the GSE62254 cohort; Fig. 3g).

### 3.4. NFκB suppresses ESRRG function in gastric cancer

*Helicobacter pylori* infection triggers inflammation and chronic infection-induced inflammation, which results in GC [23]. NF-κB κ is a well-known transcription factor that induces inflammation and promotes tumorigenesis. In the current study, we suggest that ESRRG protects cells from *H. pylori* infection. Thus, we hypothesized a functional relationship between ESRRG, TFF1, and NF-κB-induced inflammation during *H. pylori* infection. Previous reports suggest that bacterial infection activates NF-κB κ phosphorylation and translocation into the nucleus, where NF-κB functions as a transcription factor; therefore, we examined p65 (NF-κB subunit) phosphorylation in GC cells. We determined that NF-κB/p65 phosphorylation was enhanced by *H. pylori* infection and the associated CagA pathogenic protein. However, *H. pylori* infection suppressed ESRRG and TFF1 gene expression, suggesting that NF could mediate the downregulation of ESRRG by *H. pylori* κB/p65, demonstrating that ESRRG and TFF1 are inversely correlated with NF-κB/p65 (Fig. 4a and b). Upon *H. pylori* infection, NF-κB/p65 translocates to the nucleus where it plays a crucial role and co-localizes with ESRRG (Fig. 4c). Next, to investigate whether p65 directly inhibits ESRRG at the transcriptional level, we performed reporter assays using the *TFF1* promoter, ESRRG, and NFκB/p65. ESRRG-enhanced *TFF1* promoter activity was significantly inhibited by NFκB/p65 in a dose-dependent manner (Fig. 4d), suggesting that

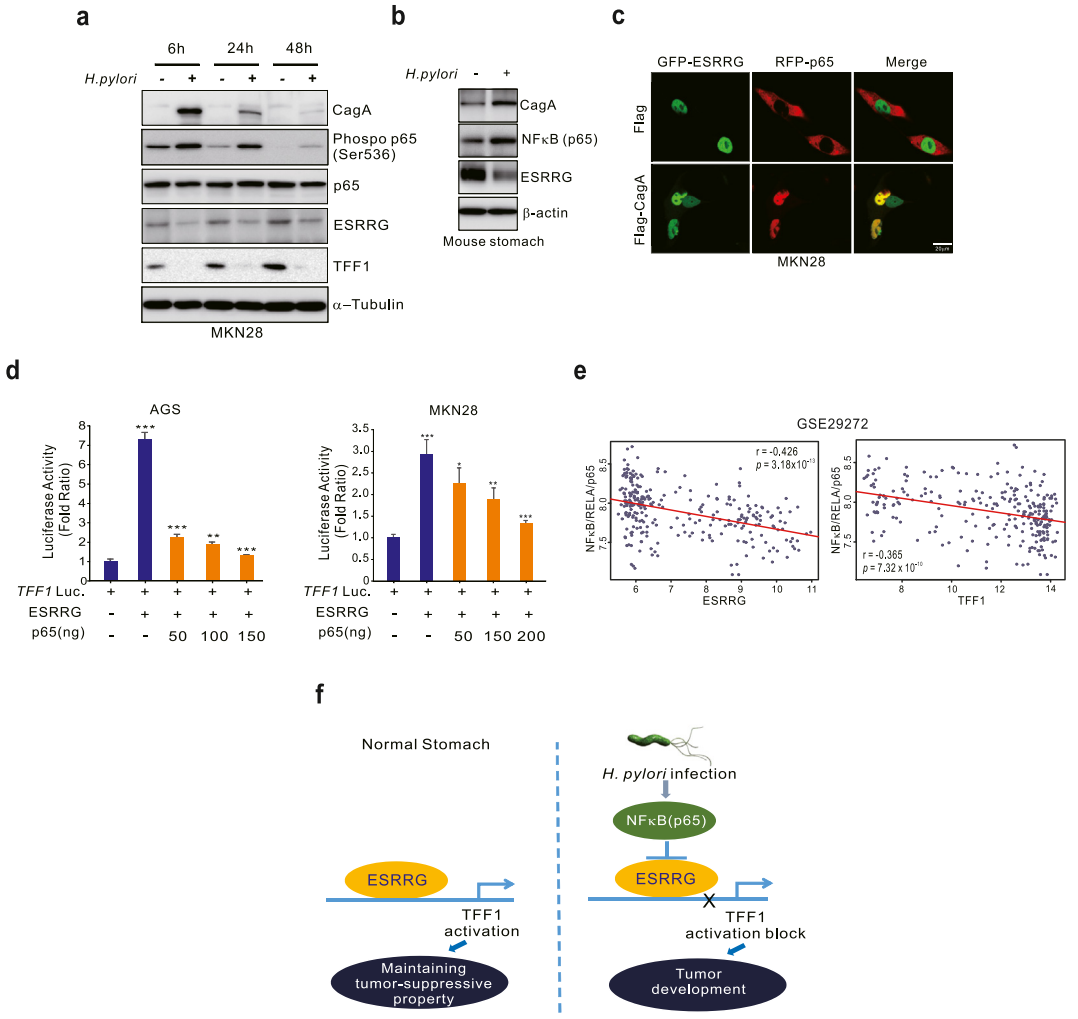


**Fig. 4.** ESRRG function is suppressed by NF-κB. (A–B) MKN28 cells (a) or mouse organoids (b) were infected with *Helicobacter pylori* for indicated durations and cells lysates were used for WB with indicated antibodies. (c) MKN28 cells were transfected with indicated constructs and used for confocal microscopy. (d) Indicated cells were transfected with indicated constructs and reporter activity was measured using a luminometer. (e) Scatter plots of ESRRG, TFF1 and NF-κB/p65/RELA expression in indicated GC patient cohorts. (f) Schematic diagram of ESRRG gene regulation during *H. pylori* infection and gastric cancer. Data represent the mean ± s.d. for indicated samples (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ by Student's *t*-test).

NFκB/p65 may suppress ESRRG-mediated TFF1 transactivation. Correlation analysis revealed that ESRRG and TFF1 were negatively correlated with NFκB/RELA in GC patients, as expected (Fig. 4e). Taken together, we demonstrated that ESRRG positively regulates TFF1 gene expression to suppress tumor development, whereas NFκB negatively regulates TFF1 by enhancing ESRRG.

## 4. Discussion

In the current study, we identified a novel tumor suppressor, ESRRG, that prevents *H. pylori* infection-induced GC. Previous reports have demonstrated that ESRRG is a tumor suppressor that regulates the Wnt pathway in GC. Although ESRRG harbors tumor suppressive properties by inhibiting the Wnt pathway, the detailed mechanisms of the contributions of ESRRG as tumor suppressors are poorly understood. Since *H. pylori* infection is a well-characterized contributing factor to GC [18], we hypothesized that ESRRG may be correlated with *H. pylori* infection. Interestingly, previously reported data demonstrated that ESRRG positively controls *Salmonella typhimurium* infection by modulating iron homeostasis in the liver [20]. Thus, GSK5182, an inverse agonist of ESRRG, can inhibit *S. typhimurium* infection in the liver, suggesting that ESRRG could be a therapeutic target for infectious diseases. Our studies suggest that ESRRG can protect gastric cells from *H. pylori* infection (Figs. 1 and 2), demonstrating that ESRRG could function in a tissue-specific manner against bacterial infection. Gene expression profiling has demonstrated that ESRRG is specifically expressed in the stomach and brain [24,25]. ESRRG overexpression itself results in a higher pH, inhibiting the survival of *H. pylori*, and thus mitigating infection, suggesting that ESRRG may promote basic conditions to inhibit *H. pylori* colonization (Figs. 1 and 2).

Gene expression profiling revealed that TFF1 is an ESRRG downstream target in GC (Figs. 3 and 4). TFF1 is a well-known tumor suppressor in GC [26]; however, TFF1 has oncogenic potential as an ERα/ESR1 and ERRα downstream target in breast cancer [27,28], suggesting a context-dependent role of estrogen-related receptors. Since TFF1 is necessary for maintaining gastric epithelial architecture, TFF1 abnormalities can lead to GC [26,29]. We found that ESRRG modulates *H. pylori* infection and cancer cell growth by regulating TFF1 gene expression. Moreover, clinical data

and experimental validation clearly suggest that TFF1 is a legitimate downstream target of ESRRG in GC and has common physiological and functional properties. Further analysis with an ESRRG conditional knockout mouse model in the stomach will be required to investigate the role of ESRRG in *H. pylori* infection and tumor progression.

Using gene expression profiling, we found that TFF1 is an ESRRG downstream target. In the current study, although we focused on TFF1 gene regulation by ESRRG, many genes were globally altered by ESRRG. While fibrinogen genes (*FGA*, *FGB*, and *FGG*) were highly upregulated by ESRRG, well-known oncogenes (*CCND1*, *BUB3*, and *RANBP1*) were downregulated by ESRRG (Fig. 3). Fibrinogen is a class of glycoproteins that are pivotal for blood coagulation. However, the involvement of FGA, FGB, and FGG in cancer is unclear. Since ESRRG is highly correlated with fibrinogen expression, it is important to investigate the functional relationship between ESRRG and fibrinogen in GC.

*Helicobacter pylori* infection induces the expression of inflammatory signaling molecules such as NF-κB and TLR4, which promote favorable conditions for cancer cell survival. The p65-NFκB subunit suppressed ESRRG gene expression via a direct interaction between p65 and the *TFF1* promoter and ESRRG (Fig. 4). Although *H. pylori* infection contributes to GC development, various oncogenic events drive this process. We suggest that ESRRG has a tumor-suppressive role by preventing *H. pylori* infection (Fig. 4f). However, ESRRG could also have a tumor-suppressive effect by controlling diverse physiological events.

## 5. Conclusion

We identified a novel role for ESRRG as a potent tumor suppressor in GC by modulating *H. pylori* infection. We also provide mechanistic insights into GC promotion and development, as well as novel therapeutic targets for the treatment of GC.

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## Author contributions

M.-H.K. and Y.-Y.P. generated the hypothesis, designed the experiments, and wrote the manuscript. M.-H.K. performed the experiments. M.-H.K. S.-i.E, and Y.-Y.P. interpreted the data.

## Declaration of competing interest

The authors declare no Conflict of Interest.

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