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Helicobacter pylori upregulates Nanog and Oct4 via Wnt/β-catenin signaling pathway to promote cancer stem cell-like properties in human gastric cancer

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ABSTRACT

Helicobacter pylori (*H. pylori*) infection is considered a major risk factor for gastric cancer. CagA behaves as a major bacterial oncoprotein playing a key role in *H. pylori*-induced tumorigenesis. Cancer stem cells (CSCs) are believed to possess the ability to initiate tumorigenesis and promote progression. Although studies have suggested that cancer cells can exhibit CSC-like properties in the tumor microenvironment, it remains unclear whether *H. pylori* infection could induce the emergence of CSC-like properties in gastric cancer cells and, the underlying mechanism. Here, gastric cancer cells were co-cultured with a CagA-positive *H. pylori* strain or a CagA isogenic mutant strain. We found that *H. pylori*-infected gastric cancer cells exhibited CSC-like properties, including an increased expression of CSC specific surface markers CD44 and Lgr5, as well as that of Nanog, Oct4 and c-myc, which are known pluripotency genes, and an increased capacity for self-renewal, whereas these properties were not observed in the CagA isogenic mutant strain-infected cells. Further studies revealed that *H. pylori* activated Wnt/β-catenin signaling pathway in a CagA-dependent manner and that the activation of this pathway was dependent upon CagA-positive *H. pylori*-mediated phosphorylation of β-catenin at the C-terminal Ser675 and Ser552 residues in a c-met- and/or Akt-dependent manner. We further demonstrated that this activation was responsible for *H. pylori*-induced CSC-like properties. Moreover, we found the promoter activity of Nanog and Oct4 were upregulated, and β-catenin was observed to bind to these promoters during *H. pylori* infection, while a Wnt/β-catenin inhibitor suppressed promoter activity and binding. Taken together, these results suggest that *H. pylori* upregulates Nanog and Oct4 via Wnt/β-catenin signaling pathway to promote CSC-like properties in gastric cancer cells.

Introduction

Gastric cancer is a leading cause of cancer-related death worldwide, and its incidence rates are highest in Eastern Asia, Central and Eastern Europe, and South America. In 2012, an estimated 951,600 new gastric cancer cases and 723,100 deaths occurred [1]. *Helicobacter pylori* (*H. pylori*) infection is the strongest identified risk factor for gastric cancer, and this bacterium has been classified as a class 1 carcinogen by the World Health Organization. A recent study has reported that approximately 89% of new cases of non-cardiac gastric cancer can be attributed to *H. pylori* worldwide [2]. The *H. pylori* genome shows genetic diversity among distinct isolates. Thus, clinically isolated *H. pylori* strains are often subdivided into two types according to the cag pathogenicity island (cag PAI)-encoded cytotoxin-associated gene A (CagA) protein. Individuals infected with CagA-positive *H. pylori* strains are at increased risk of gastric cancer compared with those with CagA-negative *H. pylori* infection [3,4]. In addition, it has been shown that CagA transgenic mice have a significantly increased incidence of gastric cancer [5,6]. The CagA protein, a major bacterial virulence factor that is injected into gastric epithelial cells via the type IV secretion system (T4SS), behaves as a bacterial oncoprotein [6]. Several different cag PAI-encoded proteins function as the component of the T4SS, including CagL, CagY, CagI and CagE, are involved in the translocation of CagA [7–9]. Moreover, *H. pylori* can exploit host cell surface molecules such as integrins and phosphatidylserine to deliver CagA to

Abbreviations: H. pylori, *Helicobacter pylori*; CSC, cancer stem cell; cag PAI, cag pathogenicity island; CagA, cytotoxin-associated gene A; T4SS, type IV secretion system; TCF/LEF, T cell factor/lymphoid enhancer factor; Oct4, octamer-binding transcription factor 4; iPS, induced pluripotent stem; WT, wild-type; RTKs, receptor tyrosine kinases; EMT, epithelial-mesenchymal transition.

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the host cells in an energy-dependent manner [10]. Once it has entered these target cells, it interacts with several host proteins, including E-cadherin, GSK3β and tyrosine kinase c-met receptor [11–13], and then continuously activates multiple oncogenic signaling pathways, including the Wnt/β-catenin, PI3K/Akt, ERK/MAPK, and JAK/STAT3 pathways [14]. Among these dysregulated oncogenic signaling pathways, the aberrant Wnt/β-catenin signaling pathway has attracted much attention and plays an essential role in tumorigenesis, including gastric cancer.

The aberrant activated Wnt/β-catenin signaling pathway has been implicated in contributing to tumorigenesis through the maintenance of cancer stem cells (CSCs) [15]. CSCs have been defined as a subset of cancer cells that possess the ability to initiate tumorigenesis. The theory of CSCs reasonably explains the heterogeneity of cells within tumors, in which a subset of stem-like cells is able to differentiate into all cell types [16]. To identify gastric CSCs, side population analyses have been conducted, and different cell-specific surface markers have been assessed. However, CD44 in particular has been identified as a marker of gastric CSCs, and it is still widely used at present [17].

β-catenin, a key component of the canonical Wnt signaling pathway, acts as a coactivator of T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors in the nucleus. In epithelial cells with insufficient Wnt stimulation, β-catenin mainly interacts with E-cadherin to form adherens junctions and free cytoplasmic β-catenin is bound by the Axin/APC/GSK3β complex, collectively termed the "destruction complex". Next, GSK3β kinase phosphorylates the N-terminus of β-catenin at the Ser33/37/Thr41 residues and targets it for degradation via the ubiquitin-proteasome system. Previous reports have indicated that (i) CagA interacts with E-cadherin and disrupts E-cadherin/β-catenin complex formation, releasing β-catenin from the E-cadherin/β-catenin compelx into the cytoplasm; and (ii) CagA binds to GSK-3β directly and depletes its activity, inhibiting the phosphorylation and proteasomal degradation of cytoplasmic β-catenin [11,12]. As a result, CagA-positive *H. pylori* induces the cytoplasmic and nuclear accumulation of β-catenin. Nuclear accumulation of β-catenin activates the Wnt signaling pathway and increases the transcriptional activity of its target genes, such as c-myc and cyclin D1. This activation has been reported to be regulated by the Akt- and/or PKA-dependent phosphorylation of β-catenin at Ser552 and Ser675 in the C-terminus. This phosphorylation increases β-catenin nuclear accumulation and enhances the recruitment of coactivators, such as TCF4 and 14-3-3ζ, to promote the transcriptional activity of target genes [18–20].

Nanog and Oct4 (octamer-binding transcription factor 4, also known as POU class 5 homeobox 1, or POU5F1), which are downstream target genes of the Wnt/β-catenin signaling pathway, are required for the maintenance of self-renewal and pluripotency of CSCs [21–23]. Additionally, Nanog and Oct4 have been identified as two of four factors essential for the reprogramming of somatic cells into induced pluripotent stem (iPS) cells [24]. Some studies have suggested that the expression of Nanog and Oct4 is increased during the dedifferentiation process of cancer cells and the oncogenic transformation of somatic cells [25,26]. Thus, Nanog and Oct4 play functional roles and are overexpressed in CSCs, and their upregulation in tumor tissues is correlated with the poor prognosis of patients with lung and oral cancers [27,28].

In this study, we found that *H. pylori* promotes CSC-like properties in gastric cancer cells via the Wnt/β-catenin signaling pathway in a CagA-dependent manner. Although some studies have confirmed that CagA-positive *H. pylori* has the ability to activate the Wnt/β-catenin signaling pathway, the underlying molecular mechanism has not been completely elucidated. We further demonstrated that the increased nuclear accumulation and transcriptional activity of β-catenin may be dependent on the CagA-positive *H. pylori*-mediated C-terminal phosphorylation of β-catenin. Additionally, we found that *H. pylori* regulates Nanog and Oct4 via the Wnt/β-catenin signaling pathway. Human gastric cancer samples from individuals infected with CagA-positive *H. pylori* exhibited increased expression of Nanog and Oct4 compared with those from individuals infected with CagA-negative *H. pylori* alone.

Materials and methods

Ethical statement and human blood/tissue samples

All experimental procedures were approved by the ethical committee of the Third Military Medical University. Written informed consent was obtained for all patient samples. All paired blood and gastric cancer tissues were collected from 29 patients with gastric cancer at Xinqiao Hospital (during 2014 and 2015). Gastric cancer diagnosis was made based on HE staining and immunophenotyping results.

Serological analysis and immunohistochemistry

All blood samples were sent to the laboratory for serum preparation, and all serum samples were detected immediately using a Typing Detection Kit for an antibody to *H. pylori* CagA (Catalog no.: CP04, Blot Biotechnology, Shenzhen, China) according to the manufacturer's protocol.

Standard ABC peroxidase techniques were used for immunohistochemistry (IHC). First, slides were incubated with an anti-Nanog (1:100 dilution, ab62734, Abcam, USA) or anti-Oct4 antibody (1:100 dilution, ab184665, Abcam, USA). Second, the primary antibody was detected using a biotinylated secondary antibody (Maxim-Bio, Fuzhou, China) and an avidin-biotin complex (Maxim-Bio), and the slides were then stained with DAB (Maxim-Bio). Finally, the slides were counterstained with Mayer's hematoxylin. We quantitatively scored tissue sections using ImagePro Plus (Media Cybernetics, USA).

Cell culture

The human gastric cancer cell line MKN45 was purchased from the Type Culture Collection of the Chinese Academy of Sciences, and the AGS cell line was purchased from the American Type Culture Collection (ATCC). MKN45 and AGS cells were cultured in RPMI 1640 medium (Catalog no.: C11875500BT, Gibco, USA) supplemented with 10% fetal bovine serum (Catalog no.: 10099141, Gibco, USA) in a humidified incubator containing 5% CO_2 at 37 °C.

H. pylori *strains and infection model*

The CagA-positive *H. pylori* strain 11637 (obtained from ATCC) and △CagA (an isogenic mutant of 11637 lacking CagA, kindly provided by Chihiro Sasakawa) were grown on brain-heart infusion plates containing 10% rabbit blood at 37 °C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2). *H. pylori* was washed from the culture plates with sterile phosphate-buffered saline (PBS) and then, centrifuged at 2500 × *g* for 5 min, and resuspended in RPMI 1640 medium without antibiotics. The amount of bacteria was determined by measuring optical density at 600 nm (1 OD600 = 1×10^9 CFU/ml). RPMI 1640 medium alone served as a blank control.

Cultured cells were seeded on plates and grown to 80% confluency. Then, *H. pylori* was added to cells at a bacteria-to-cell ratio of 100:1 (multiplicity of infection, MOI = 100).

Flow cytometry analysis

Cells were washed once with PBS, and they were then dissociated from the plates using Trypsin-EDTA (Catalog no.: T4049, Sigma, USA) and centrifuged. Cell pellets were resuspended and stained with anti-CD44-FITC (Clone G44-26; BD Pharmingen, USA), anti-CD24-PE antibodies (Clone ML5; BD Pharmingen, USA), anti-Lgr5-PE antibodies (Clone 8F2; BD Pharmingen, USA) and anti-CD133-PE antibodies (Clone AC133; Miltenyi Biotec, GER) in a solution containing PBS, 0.5% bovine serum albumin, 2 mmol/L EDTA for 30 min at 4 °C. Finally, the samples were analyzed by flow cytometry (BD FACSAria II, CA).

Spheroid formation assay

For spheroid formation assay, cells were recovered and 1000 cells were seeded on non-adherent 24-well culture plates (Costar, Catalog no.: 3473; Corning Inc., Corning, NY) in stem cell medium, i.e. serum free DMEM/F12 medium (Catalog no.: C11320500BT, Gibco, USA) containing 40 ng/ml epidermal growth factor (EGF, Catalog no.: AF10015, PeproTech Inc., Rocky Hill, NJ), 20 ng/ml basic fibroblast growth factor (basic-FGF, Catalog no.: 10018B, PeproTech Inc., Rocky Hill, NJ) and B27 supplement (1X, Catalog no.: 17504044, Gibco, USA). After 3 days, an equal amount of fresh medium was added. Cells were then incubated for 7 days, and spheroids with a diameter of >75 μm were counted under a light microscope.

Table 1
Primer sequences used for real-time PCR.

Gene	Sequence
CD44	F: TGCCGCTTTGCAGGTGTAT
	R: GGCCTCCGTCCGAGAGA
CD24	F: GACATGGGCAGAGCAATGGTGGC
	R: GAGTGAGACCACGAAGAGACTGGC
Lgr5	F: TATGCCTTTGGAAACCTCTC
	R: CACCATTCAGAGTCAGTGTT
Nanog	F: ACCTATGCCTGTGATTTGTGG
	R: AGTGGGTTGTTTGCCTTTGG
Oct4	F: TCAGCTTCCTCCACCCACTT
	R: TATTCAGCCAAACGACCATCT
actin	F: ACAGAGCCTCGCCTTTGC
	R: GCGGCGATATCATCATCC

Quantitative real-time PCR

Total RNA was extracted using a TRIZOL Reagent Kit (Catalog no.: 15596026, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of the RNA using random primer according to the manufacturer's protocol of a PrimeScript RT Reagent Kit (Catalog no.: RR047A, TaKaRa, Ohtsu, Japan), and quantitative real-time PCR was performed using SYBR Green Mix (Catalog no.: RR820A, TaKaRa). The detail of reverse transcription and PCR procedures was provided in Supplementary Materials and Methods. The primers used were synthesized by Sangon Biotech (Shanghai, China) and are listed in Table 1. The results were normalized to the level of β-actin. Each reaction was performed in triplicate.

Western blotting

After cells were lysed, protein concentrations were measured using a BCA Protein Assay Kit (Catalog no.: 23250, Thermo, USA) and western blotting analyses were performed as previously described [29]. Primary anti-Nanog (ab184609), anti-Oct4 (ab184665), anti-c-myc (ab39688), anti-Sox2 (ab184529), anti-Klf4 antibodies (ab155808) were purchased from Abcam (Cambridge, MA, USA); anti-β-catenin (#8480), anti-p-β-catenin (Ser675) (#4176), anti-p-β-catenin (Ser552) (#5651), anti-E-cadherin (#3195), anti-N-cadherin (#13116), anti-vimentin antibodies (#5741) were purchased from Cell Signal Technology (CST, USA); and anti-CagA (sc-28368), anti-snail (sc-28199), anti-GAPDH (sc-25778), anti-tubulin (sc-58886) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated secondary goat-rabbit (#ZB-2305) and goat-mouse (#ZB-2301) IgG antibodies were purchased from Zhong Shan Gold Bridge Biotechnology (Beijing, China). An ECL chemiluminescence kit was purchased from Thermo (Catalog no.: 34095, USA).

Immunofluorescence

Cells were seeded on coverslips and grown to 80% confluency, and they were then infected with *H. pylori* for 24 h. Next, the cells were washed once with PBS, and then fixed in 4% paraformaldehyde for 10 min at room temperature and solubilized in PBS-0.5% Triton-X100 for 15 min at room temperature. The cells were then incubated with 5%BSA for 30 min at room temperature. Next, they were incubated with primary anti-β-catenin antibody (#8480, CST, USA) overnight at 4 °C and subsequently with the secondary antibodies Alexa Fluor 647 goat-anti mouse IgG and Cy3-conjugated goat anti-rabbit IgG (each at a 1:100 dilution) for 1 h. DAPI was used to stain cell nuclei.

Reagents and Luciferase reporter vectors

The following reagents were used: Wnt/β-catenin inhibitor, xav939 (sc-296704, Santa Cruz, CA, USA), c-met kinase inhibitor, SU11274 (sc-204801, Santa Cruz, CA, USA), PI3K/Akt inhibitor, and GSK 690693 (sc-363280, Santa Cruz, CA, USA). Top-Flash (catalog no. 21–170, 5.5 kb) and Fop-Flash (catalog no. 21–169, 5.5 kb.) were purchased from Millipore. Construction of luciferase reporter vectors was performed as previously described [29]. Briefly, the promoters of Nanog and Oct4 were amplified from human cDNA. The amplified wild-type Nanog fragment was cloned into the KpnI and BglII sites of a PGL3-basic vector (Catalog no.: E1751, Promega, USA), and the amplified wild-type Oct4 fragment was cloned into the KpnI and XhoI sites of the PGL3-basic vector. The detail of reaction conditions and the result of identification were provided in Supplementary materials and methods.

Transfection and dual-luciferase assay

Cells were starved for 1 h and were then transfected with luciferase reporter vectors. Lipofectamine 2000 (Catalog no.: 11668-019, Invitrogen, Carlsbad, CA, USA) was used to transfect MKN45 cells and X-tremeGENE HP DNA Transfection Reagent (Catalog no.: 06366546001, Roche, Switzerland) was used to transfect AGS cells.

Cells were seeded in 96-well plates, and cells at approximately 80% confluence were transfected with the constructed luciferase reporter vectors for 4 h. Transfection mixtures were then replaced with complete medium. Next *H. pylori* was added to the cells at a bacteria-to-cell ratio of 100:1. After 24 h, the cells were harvested in 1X Reporter Lysis Buffer (Catalog no.: E1960, Promega). Luciferase activity was determined as the average of three independent assays using a dual-luciferase assay system (Catalog no.: E1960, Promega). Luciferase activity was normalized to Renilla luciferase activity.

Chromatin immunoprecipitation (ChIP) assays

ChIP assay was performed following the manufacturer's instructions (Millipore) as previously described [29]. Briefly, chromatin was isolated from MKN45 cells infected with *H. pylori* or treated with xav939 for 24 h. The cells were then fixed for 10 min with 1% formaldehyde and disrupted in EZ-Zyme™ lysis buffer. Lysates were snap frozen in liquid N_2 and then thawed in 37 °C water to cleave chromatin. One part of chromatin was stored as a positive control for subsequent experiments ("Input" team), and another party of chromatin was immunoprecipitated with 2 μg IgG as a negative control ("IgG" team). The rest of chromatin was immunoprecipitated with an antibody (2 μg) directed against β-catenin. The final DNA extracts were PCR amplified.

Statistical analysis

Quantitative data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 6.0 software (San Diego, CA, USA). Unpaired Student's t test was used to compare two groups, and one-way ANOVA was used to compare three or more groups. $P < 0.05$ was considered statistically significant.

Results

CagA-positive H. pylori*-infected gastric cancer cells exhibit cancer stem cell-like properties*

CSCs express specific surface markers and possess the capacity for self-renewal. Thus, surface markers are widely used to identify CSCs, and the spheroid formation assay is frequently performed to assess the capacity of CSCs for self-renewal [17,30]. To examine whether CagA-positive *H. pylori*-infected gastric cancer cells exhibit CSC-like properties, we first infected gastric cancer cells with bacterial strain 11637 (CagA-positive *H. pylori*) or an isogenic mutant of 11637 lacking CagA (11637 △CagA). The number of CD44-positive gastric cancer cells was significantly increased after 11637 wild-type (WT) strain infection, while it was slightly but not significantly increased after 11637 △CagA strain infection (Fig. 1A). Leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5), initially considered as gastric and intestinal stem cell marker and gradually regarded as CSC marker in cancer models [31–33], was also evaluated and showed significant increase in 11637 WT strain-infected gastric cancer cells but not in uninfected and 11637 △CagA strain-infected gastric cancer cells (Supplementary Fig. S1A). However, CD24 and CD133, another CSC markers in gastrointestinal cancer [34,35], was also evaluated but showed no significant change in gastric cancer cells in response to *H. pylori* infection (Fig. 1A and Supplementary Fig. S1B). The result of CD24 is in agreement with those of Bessede et al. who reported that CD24 expression level was not affected by *H. pylori* infection in AGS cells [36]. Furthermore, our results indicated that CD133 expression was too low (less than 1%) in gastric cancer cells, which is consistent with the report of Zhang et al. in the same cell lines [35]. Recently, Su et al. reported CD133 contributes to maintenance of CSC properties in cell lines from brain, colon and lung cancers, but not gastric or breast cancers [37].

Then we performed qRT-PCR to detect the mRNA expression of CD44 and Lgr5 in *H. pylori*-infected gastric cancer cells. Consistent with membrane-expressed CD44 and Lgr5, the mRNA level of CD44 and Lgr5 was significantly increased after 11637 WT strain infection but not after 11637 △CagA strain infection (Fig. 1B and Supplementary Fig. S1C). Next, spheroid formation assay was performed in vitro under non-adherent culture conditions. After

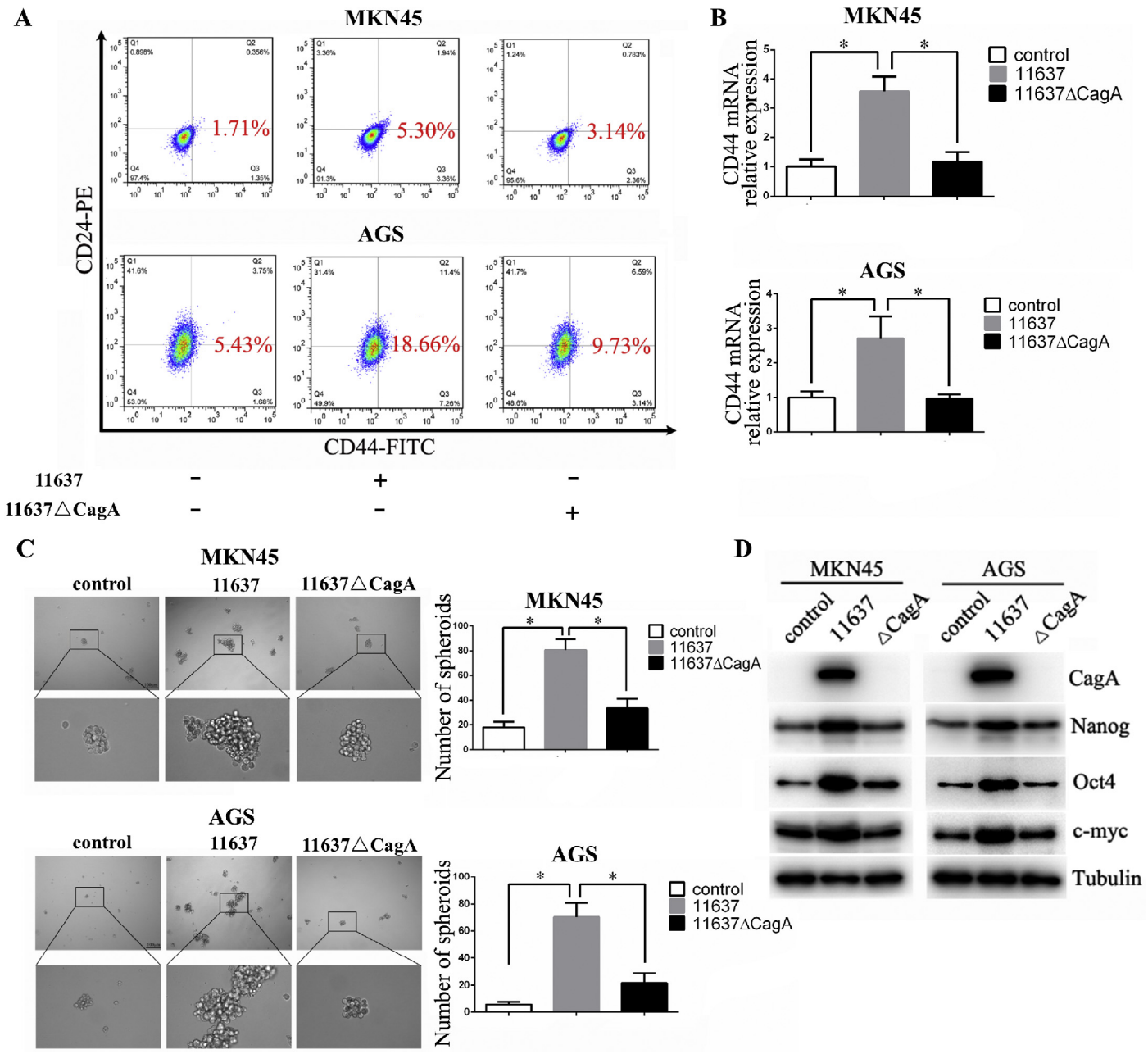

Fig. 1. CagA-positive *H. pylori*-infected gastric cancer cells exhibit cancer stem cell-like properties. After infection with the *H. pylori* 11637 WT or 11637 △CagA strain for 24 h, MKN45 and AGS cells were harvested. (A) The percentage of CD44-positive cells was quantified using flow cytometry in *H. pylori* 11637 WT-infected cells vs uninfected cells (control) or 11637 △CagA strain-infected cells. (B) The relative expression of CD44 mRNA was measured by PCR. (C) The harvested cells were seeded under non-adherent culture conditions for spheroid formation. The number of spheroids was quantified after 7 days. (D) The Nanog, Oct4 and c-myc protein levels were assayed by western blotting.

infection with the 11637 WT or 11637 △CagA strain for 24 hours, gastric cancer cells were cultured in serum-free medium under non-adherent conditions. Gastric cancer cells infected with the11637 WT strain showed a significantly increased spheroid formation compared with uninfected control cells or 11637 △CagA strain-infected cells (Fig. 1C). Moreover, the spheroids in gastric cancer cells infected with the 11637 WT strain were much larger than those in uninfected control cells or 11637 △CagA strain-infected cells, as shown in Fig. 1C. Notably, core transcription factors (such as Nanog, Oct4 and c-myc), which play functional roles and are overexpressed in CSCs, are being increasingly used as markers for identifying CSCs [21–23,38]. Therefore, we examined several core transcription factors, including Nanog, Oct4, c-myc, Sox2 and Klf4, in both uninfected control gastric cancer cells and in *H. pylori* (11637 WT or 11637 △CagA strain)-infected gastric cancer cells. Although the expression of Sox2 and Klf4 did not change after *H. pylori* infection in gastric cancer cells (Supplementary Fig. S1D), that of Nanog, Oct4 and c-myc waw significantly increased after 11637 WT strain infection but not after 11637 △CagA strain infection (Fig. 1D). These data indicate that CagA-positive *H. pylori*-infected gastric cancer cells exhibit CSC-like properties. However, cells infected with the CagA-mutant strain did not exhibit CSCs-like properties, indicating that the enhancement of CSCs-like properties is dependent upon CagA.

CagA-positive H. pylori *activates Wnt/β-catenin signaling, including β-catenin nuclear accumulation and transcriptional activation*

The Wnt/β-catenin signaling pathway, which is closely involved in the regulation of CSCs, has been implicated in gastrointestinal tumorigenesis via its function in CSCs [15,39]. Therefore, we hypothesized that *H. pylori* may promote CSC-like properties in cancer cells via activation of the Wnt/β-catenin signaling pathway. Because previous studies have reported that CagA-positive *H. pylori* activates Wnt/β-catenin signaling [11,12,40], we first re-evaluated the capacity of our bacterial strain to promote this signaling pathway. As expected, infection of gastric cancer cells with the 11637 WT strain, but not with the 11637 △CagA strain, resulted in nuclear accumulation of β-catenin (Fig. 2A). Notably, its nuclear accumulation was observed after 11637 WT strain infection, regardless of whether β-catenin was localized to the cytomembrane in MKN45 cells or to the cytoplasm in AGS cells (Fig. 2A). Next, we performed a luciferase reporter assay to determine the functional consequences of the CagA-positive *H. pylori*-induced nuclear translocation of β-catenin. This assay revealed that the 11637 WT strain enhanced luciferase activity, while the 11637 △CagA strain did not (Fig. 2B). These data indicate that the 11637 WT strain induces the nuclear accumulation and transcriptional activation of β-catenin and that this process is dependent upon CagA. Although previous reports have indicated CagA induces the cytoplasmic accumulation of β-catenin by disrupting E-cadherin/β-catenin complex formation or by depleting GSK-3β activity [11,12], the molecular mechanisms of β-catenin nuclear accumulation and transcriptional activation have not been completely elucidated during CagA-positive *H. pylori* infection.

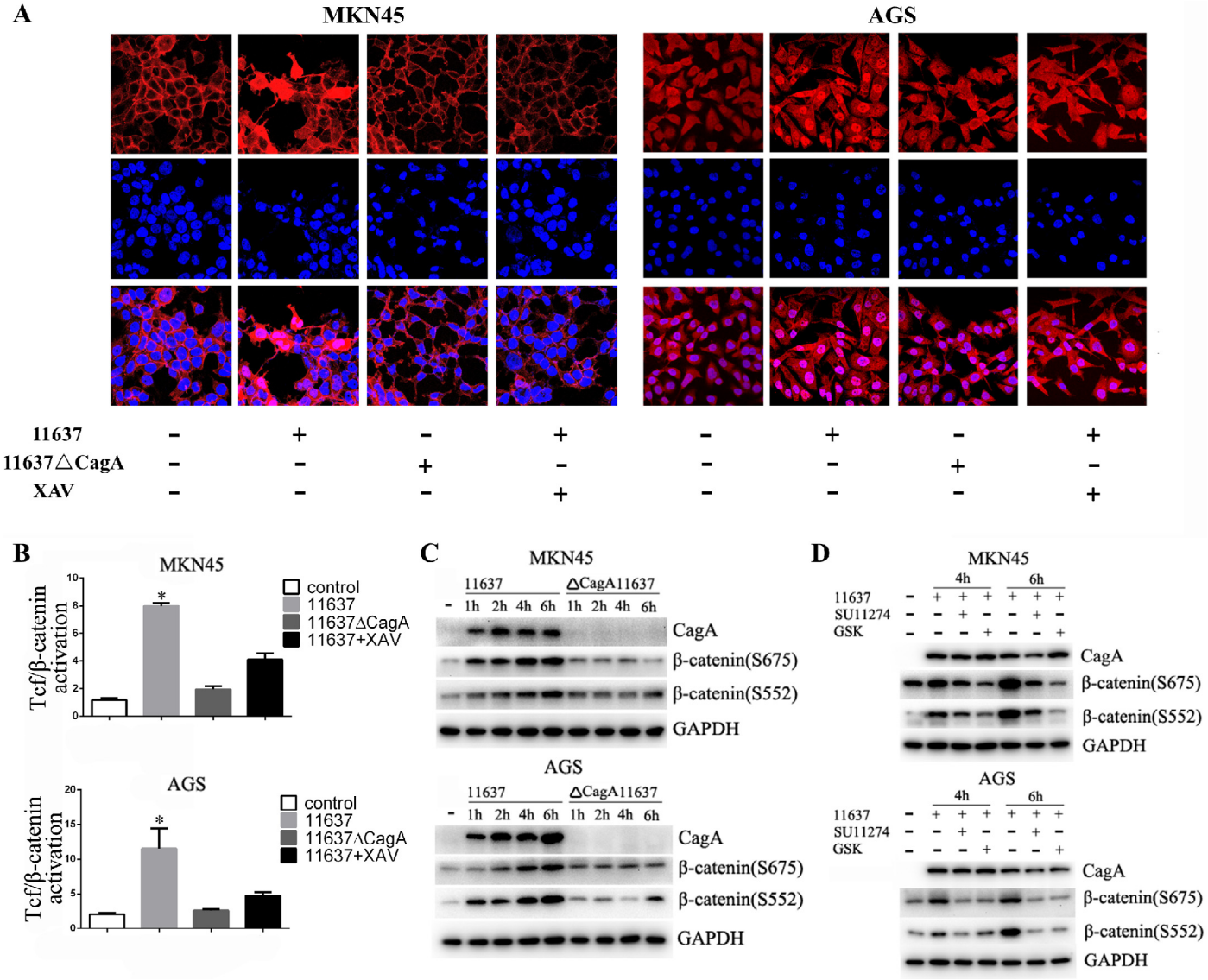

Fig. 2. CagA-positive *H. pylori* activates Wnt/β-catenin signaling, including β-catenin nuclear accumulation and transcriptional activation. (A) MKN45 and AGS cells were infected with *H. pylori* (11637 WT or 11637 △CagA strain), or pre-treated with an inhibitor of Wnt/β-catenin (XAV, 10 μM) for 1 h, and they were then infected with *H. pylori* 11637 WT for 24 h. The localization of β-catenin was observed via immunofluorescence. (B) MKN45 and AGS cells were pre-transfected with luciferase reporter constructs containing LEF/TCF-binding motifs (Top-Flash) or mutated LEF/TCF sites (Fop-Flash) for 4 h. Luciferase activity was measured to assess β-catenin transcriptional activity in uninfected, 11637 WT-infected, 11637 △CagA strain-infected and XAV pre-treated 11637 WT-infected cells after 24 h. (C) Western blotting was used to assess β-catenin phosphorylation (Ser675 and Ser552) in MKN45 and AGS cells after *H. pylori* 11637 WT or 11637 △CagA strain infection for different times. (D) MKN45 and AGS cells were pre-treated with a c-met inhibitor (SU11274, 5 μM) or a PI3K/Akt inhibitor (GSK, 5 μM) and then infected with *H. pylori* 11637 WT. Western blotting was performed to assay β-catenin phosphorylation (Ser675 and Ser552) after the treatment.

Previous studies have demonstrated that the phosphorylation of Ser675 and Ser552 in the C-terminus of at β-catenin promotes its nuclear accumulation and transcriptional activity, resulting in increased activation of the Wnt/β-catenin signaling pathway [18,41]. Therefore, we also evaluated the levels of phosphorylated β-catenin in uninfected control and *H. pylori*-infected gastric cancer cells. Indeed, the 11637 WT strain significantly promoted the phosphorylation of β-catenin at Ser675 and Ser552 in a time-dependent manner, whereas the 11637 △CagA strain did not (Fig. 2C). As phosphorylation of β-catenin is well known to be regulated by receptor tyrosine kinases (RTKs) c-met and its downstream Akt, gastric cancer cells were treated with c-met the kinase inhibitor SU11274 and the Akt inhibitor GSK before *H. pylori* infection. As shown in Fig. 2D, inhibition of c-myc and Akt separately induced a significant reduction in the *H. pylori*-induced phosphorylation of β-catenin at Ser675 and Ser552 in both MKN45 and AGS cells. These results suggest that c-myc and Akt are involved in the CagA-positive *H. pylori*-induced phosphorylation of β-catenin.

Activation of Wnt/β-catenin signaling is responsible for H. pylori*-induced cancer stem cell-like properties*

To investigate whether the activation of Wnt/β-catenin signaling pathway is responsible for *H. pylori*-induced CSC-like properties, MKN45 and AGS cells were treated with the Wnt/β-catenin signaling inhibitor XAV before *H. pylori* infection. Indeed, XAV inhibited CagA-positive *H. pylori*-induced nuclear accumulation and the transcriptional activity of β-catenin, indicating that the activation of Wnt/β-catenin signaling was inhibited (Fig. 2A and B). Next, we performed flow cytometry analysis to compare the percentages of CD44-positive gastric cancer cells treated with XAV versus those not treated followed by *H. pylori* infection. Treatment with XAV resulted in a reduction in the percentage of 11637 WT strain-induced CD44-positive gastric cancer cells for both MKN45 and AGS cells (Fig. 3A). Similarly, the mRNA expression of CD44 in 11637 WT strain-infected MKN45 and AGS cells was also inhibited by XAV (Fig. 3B). These results suggest that Wnt/β-catenin signaling has an important role in the CagA-positive *H. pylori*-induced upregulation of CD44-positive gastric cancer cells. Next, we investigated the effect of XAV on CagA-positive *H. pylori*-induced spheroid formation in MKN45 and AGS cells. As expected, XAV significantly inhibited their formation (Fig. 3C). These results suggest that activation of the Wnt/β-catenin signaling pathway is responsible for *H. pylori*-induced cancer stem cell-like properties.

Wnt/β-catenin signaling activation is involved in H. pylori*-induced upregulation of Nanog and Oct4*

As shown in Fig. 1D, Nanog and Oct4 expression was upregulated in CagA-positive *H. pylori*-infected MKN45 and AGS cells. Thus, to investigate whether the activation of Wnt/β-catenin signaling could affect Nanog and Oct4 expression during CagA-positive *H. pylori* infection, XAV was used to inactivate Wnt/β-catenin signaling. As shown in Fig. 4A, XAV significantly inhibited the upregulation of Nanog and Oct4 induced by the 11637 WT strain in MKN45 and AGS cells, indicating that the activation of Wnt/β-catenin signaling is involved in the CagA-positive *H. pylori*-induced upregulation of Nanog and Oct4. Moreover, the expression of Nanog and Oct4 mRNA was increased after 11637 WT strain infection but not after 11637 △CagA strain infection. The upregulation of Nanog and Oct4 was also inhibited by XAV, indicating that *H. pylori* regulates Nanog and Oct4 at the transcriptional level and that the CagA-positive *H. pylori*-induced activation of Wnt/β-catenin signaling is involved in this process (Fig. 4B). We then constructed two vectors containing either the promoter of Nanog or the promoter of Oct4. Luciferase reporter assay revealed that the 11637 WT strain enhanced the promoter activities of Nanog and Oct4, whereas XAV decreased this 11637 WT strain-enhanced promoter activity (Fig. 4C). This findings indicated that CagA-positive *H. pylori* may promote the binding of β-catenin to the promoters of Nanog and Oct4. As revealed in Fig. 4D, a potential β-catenin-binding site (comprising the sequence: GTCTGGGT) is present in the Nanog promoter, and two potential binding sites are present in the Oct4 promoter [22,42]. Subsequently, ChIP assay was performed to examine β-catenin binding sites (BSs). As shown in Fig. 4E, the 11637 WT strain markably induced β-catenin binding to the BS in the promoter of Nanog, whereas the 11637 △CagA strain did not, and XAV inhibited this binding. Meanwhile, the 11637 WT strain only induced β-catenin binding to BS1 element (sequence: AACAAAG) in the promoter of Oct4. These results demonstrate that CagA-positive *H. pylori* promotes Nanog and Oct4 expression through the binding of β-catenin to their promoters.

CagA-positive H. pylori *infection increases Nanog and Oct4 expression in gastric cancer tissues*

Considering the ability of CagA-positive *H. pylori* to upregulate Nanog and Oct4 expression in vitro in gastric cancer cells, we sought to determine whether CagA-positive *H. pylori* infection increases Nanog and Oct4 expression in gastric cancer tissues. First, we performed the ^{13}C urea breath test to investigate whether patients diagnosed with gastric cancer were infected with *H. pylori*. The prevalence of *H. pylori* in these patients was 96.55% (28/29), which is consistent with previous reports. To further distinguish between the CagA-positive and CagA-negative patients, we collected blood samples from these patients for detection of anti-CagA antibody (28 cases, Supplementary Fig. S3A). Eighteen were found to be infected with CagA-positive *H. pylori*, and the remaining ten were found to be infected with CagA-negative *H. pylori*. Then, we performed qRT-PCR and immunohistochemistry to examine the relationship between *H. pylori* infection and Nanog and Oct4 expression in the tissues of the gastric cancer patients. As shown in Fig. 5A, 5B and Table 2, significantly increased expression of Nanog and Oct4 protein was detected in the gastric cancer samples in association with CagA-positive *H. pylori* infection compared with CagA-negative infection. Moreover, the relative mRNA expression of Nanog and Oct4 were also increased in gastric cancer tissues from CagA-positive *H. pylori*-infected patients compared with CagA-negative *H. pylori*-infected patients (Fig. 5C).

Discussion

In this study, we have shown that CagA-positive *H. pylori*-infected gastric cancer cells exhibit CSC-like properties. Previous studies have suggested that the epithelial-mesenchymal transition (EMT) is a developmental process during which epithelial cells acquire stem cell-like properties [43]. Recent evidence suggests that *H. pylori* infection induces the EMT, which may play a potential role in the emergence of gastric CSCs [36,44]. Thus, we evaluated the capacity of our bacterial strain to induce the EMT. Infection with the 11637 WT strain resulted in the emergence of EMT-like phenotype, whereas this phenotype was not observed with 11637 △CagA strain (Supplementary Fig. S2A). Decreased expression of the epithelial marker E-cadherin, along with increased expression of the mesenchymal markers N-cadherin, Vimentin and Snail, were also observed in 11637 WT strain-infected gastric cancer cells (Supplementary Fig. S2B). These findings indicate that the enhanced CSC-like properties are coincident with the EMT phenotype following CagA-positive *H. pylori* infection. However, this evidence is still insufficient to confirm that CagA-positive *H. pylori*-infected gastric cancer cells exhibit CSC-like properties via the EMT, as there is no direct evidence that suppression of the EMT inhibits

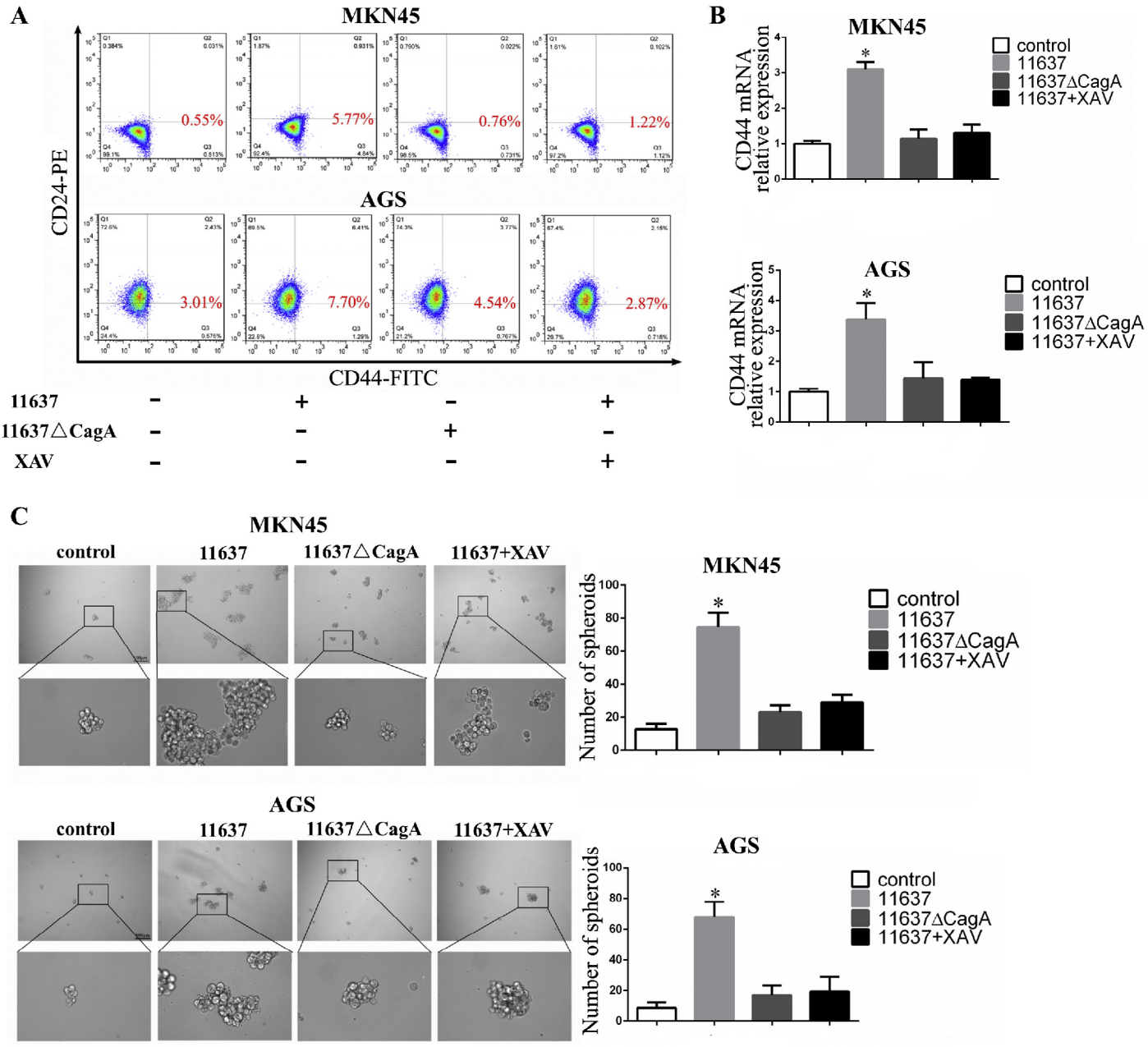

Fig. 3. The activation of Wnt/β-catenin signaling is responsible for *H. pylori*-induced cancer stem cell-like properties. MKN45 and AGS cells were infected with *H. pylori* (11637 WT or 11637 △CagA strain), or pre-treated with an inhibitor of Wnt/β-catenin (XAV, 10 μM) for 1 h, and they were then infected with *H. pylori* 11637 WT for 24 h. (A) The percentage of CD44-positive cells was quantified by flow cytometry. (B) The relative expression of CD44 mRNA was measured by PCR. (C) Uninfected cells, *H. pylori*-infected cells or XAV pre-treated *H. pylori*-infected cells were seeded under non-adherent culture conditions for spheroid formation. The number of spheroids was quantified after 7 days.

CagA-positive *H. pylori*-induced CSC-like properties in these cells. In addition, *H. pylori* may promote the self-renewal of a handful of CSCs to exhibit CSC-like properties in gastric cancer cells. A recent study has reported that *H. pylori* has the ability to activate and expand gastric stem cells through direct colonization in the middle of gastric glands, where an abundance of these gastric stem cells are present [45]. Although CagA is not required for gland colonization, it contributes to accelerated proliferation of gastric stem cells [45]. These results imply that CagA-positive *H. pylori* may enhance CSC-like properties via EMT-like changes or the acceleration of CSC self-renewal. The mechanism of the *H. pylori*-induced EMT has been clarified as follows: (i) *H. pylori* induces the EMT in gastric cancer cells, partially mediated by a cooperative network involving *H. pylori*-induced MMP-7, HB-EGF and gastrin upregulation [46]; (ii) *H. pylori* secretes large amounts of tumor necrosis factor-α-inducing protein (Tipα), which induces the EMT by directly binding to nucleolin on the cell surface to promote the expression of TNF-α and activation of NF-κB [47]; (iii) *H. pylori* CagA induces the snail-mediated EMT via the depletion of GSK-3β [12]; (iv) *H. pylori* CagA induces the twist1-mediated EMT via the inhibition of programmed cell death factor 4 [48]. However, the mechanism of *H. pylori*-induced CSC self-renewal has not been clarified.

Wnt/β-catenin signaling plays an essential role in the maintenance of CSC properties, including self-renewal and specific marker expression. Activation of Wnt/β-catenin signaling accelerates the self-renewal of CSCs and upregulates the expression of CSC markers,

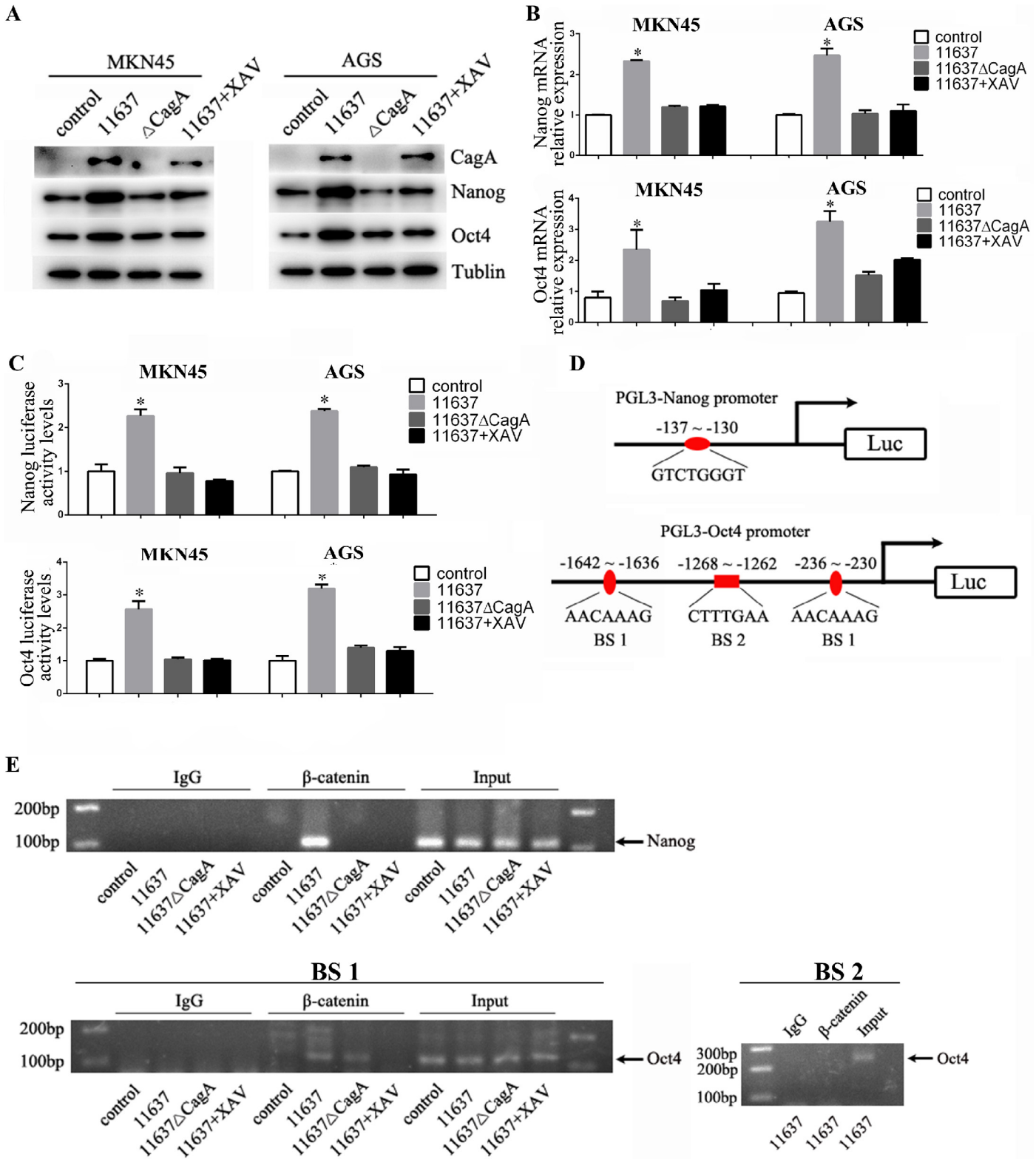

Fig. 4. Wnt/β-catenin signaling activation is involved in the *H. pylori*-induced upregulation of Nanog and Oct4. Nanog and Oct4 expression were assayed by western blotting (A) and PCR (B) in uninfected, 11637 WT-infected, 11637 △CagA strain-infected or XAV pre-treated 11637 WT-infected MKN45 and AGS cells. (C) MKN45 and AGS cells were pre-transfected with luciferase reporter constructs containing the Nanog promoter or the Oct4 promoter for 4 h. Luciferase activity was measured to assess promoter activity in uninfected, 11637 WT-infected, 11637 △CagA strain-infected and XAV pre-treated 11637 WT-infected cells after 24 h. (D) The potential binding sites for β-catenin in the promoters of Nanog and Oct4. (E) ChIP assays were performed using chromatin isolated from in uninfected, 11637 WT-infected, 11637 △CagA strain-infected or XAV pre-treated 11637 WT-infected MKN45 cells. Final DNA extracts were PCR-amplified using primers that covered the potential binding sites in the Nanog and Oct4 promoter regions. "IgG" team means negative control, and "Input" team means positive control.

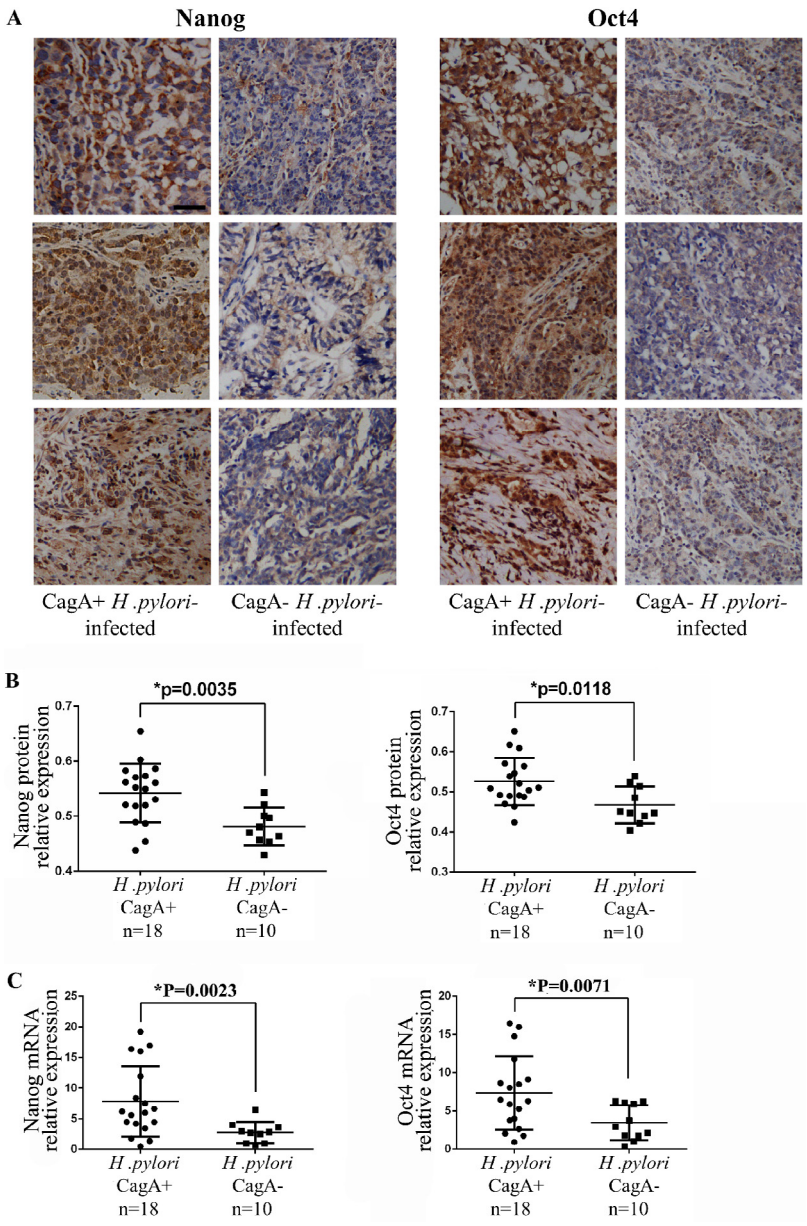

Fig. 5. CagA-positive *H. pylori* infection increases Nanog and Oct4 expression in gastric cancer tissues. (A) Representative immunohistochemical staining for Nanog and Oct4 in gastric cancer tissues from CagA-positive *H. pylori*-infected and CagA-negative *H. pylori*-infected patients. (B) The relative Nanog and Oct4 staining intensity in gastric cancer tissues from CagA-positive *H. pylori*-infected (n = 18) and CagA-negative *H. pylori*-infected patients (n = 10). The detail of statistics results were showed in Table 2. (C) The relative mRNA expression of Nanog and Oct4 in gastric cancer tissues from CagA-positive *H. pylori*-infected and CagA-negative *H. pylori*-infected patients.

whereas blockade of this signaling suppresses CSC properties [49,50]. In the tumor metabolic microenvironment, chronic metabolic stress could cause cancer cells to exhibit CSC-like properties via activation of Wnt/β-catenin [51]. In this study, we found that CagA-positive *H. pylori* was able to activate the Wnt/β-catenin signaling pathway. The nuclear accumulation and transcriptional activity of β-catenin are increased during CagA-positive *H. pylori* infection (Fig. 2A and B). Blockade of Wnt/β-catenin signaling suppresses CagA-positive *H. pylori*-induced CSC-likes properties (Fig. 3). Therefore, activation of the Wnt/β-catenin signaling pathway is responsible for the emergence of *H. pylori*-induced CSC-like properties.

Phosphorylation of β-catenin affects its stabilization, localization and function. It is well known that β-catenin protein stability is controlled by the GSK3β-mediated phosphorylation of β-catenin at Ser33/37/Thr41 and subsequent its proteasomal degradation, whereas the GSK3β-mediated phosphorylation of β-catenin at Ser552 results in its nuclear accumulation and transcriptional activation [18]. Additionally, the RTK-mediated phosphorylation of β-catenin at Tyr654 results in its release from cadherin and an increase in its PKA-mediated phosphorylation of at Ser675. Thus, enhanced Tyr654 and Ser675 phosphorylation of β-catenin has been suggested to increase its nuclear accumulation and transcriptional activation [41]. In this study, we found that CagA-positive *H. pylori* promoted the phosphorylation of β-catenin at Ser552 and Ser675 in a time-dependent manner (Fig. 2C). These results could partly explain the *H. pylori*-induced nuclear accumulation and transcriptional activation of β-catenin. Furthermore, the phosphorylation of β-catenin at Ser552 and Ser675 is correlated with c-met and its downstream molecule Akt. Previous studies have indicated that *H. pylori* CagA is involved in the interaction with c-met and the activation of Akt [13,52]. Therefore, we used SU11274 and GSK to inhibit the activation of c-met and Akt separately. As shown in Fig. 2D, the

Table 2
The detail of statistics results of Nanog and Oct4 expression in gastric cancer tissues.

CagA+ *H. pylori*	Nanog	Oct4	CagA– *H. pylori*	Nanog	Oct4
Sample 1	0.58311	0.61733	Sample 1	0.50059	0.44737
Sample 2	0.55272	0.60912	Sample 2	0.46410	0.42163
Sample 3	0.60307	0.65166	Sample 3	0.45629	0.40370
Sample 4	0.57332	0.53900	Sample 4	0.42897	0.52391
Sample 5	0.56091	0.54674	Sample 5	0.48063	0.53887
Sample 6	0.48937	0.57061	Sample 6	0.45311	0.51433
Sample 7	0.52096	0.50877	Sample 7	0.49688	0.48553
Sample 8	0.65491	0.42399	Sample 8	0.52119	0.43989
Sample 9	0.45387	0.48984	Sample 9	0.54322	0.45119
Sample 10	0.56192	0.49118	Sample 10	0.46980	0.44706
Sample 11	0.52007	0.50397			
Sample 12	0.58674	0.52099			
Sample 13	0.48680	0.56431			
Sample 14	0.51877	0.51090			
Sample 15	0.54989	0.46355			
Sample 16	0.53062	0.47001			
Sample 17	0.43842	0.49213			
Sample 18	0.57099	0.48766			

CagA-positive *H. pylori*-induced phosphorylation of β-catenin at Ser552 and Ser675 was significantly inhibited by treatment with SU11274 or GSK. Thus, CagA-positive *H. pylori* induces the phosphorylation of β-catenin at Ser552 and Ser675 in a c-met- and/or Akt-dependent manner, resulting in β-catenin nuclear accumulation and transcriptional activation.

Nanog and Oct4 are downstream target genes of Wnt/β-catenin signaling, and those proteins have been suggested to be critical regulators of CSC self-renewal and pluripotency [38,53]. Moreover, Nanog and Oct4 have also been implicated in promoting the EMT [27,54]. Consistent with these findings, the elevated expression of Nanog and Oct4 has been associated with the late-stage progression and poor prognosis of patients with cancer [27,28,38]. In this study, we found that CagA-positive *H. pylori* upregulated Nanog and Oct4 through activation of Wnt/β-catenin signaling (Fig. 4A and B). To further confirm the role of β-catenin in the *H. pylori*-induced upregulation of Nanog and Oct4, we performed luciferase reporter and ChIP assays to confirm that β-catenin binds to the –137 ~ –130 bp region of the Nanog promoter and to the –236 ~ –230 bp/–1642 ~ –1632 bp regions of the Oct4 promoter (Fig. 4C and D). In addition, the clinical data revealed that Nanog and Oct4 were significantly increased in the gastric cancer samples from CagA-positive *H. pylori*-infected patients compared with those from the CagA-negative *H. pylori*-infected patients (Fig. 5B and C). To our knowledge, this study is the first to demonstrate that CagA-positive *H. pylori* activates Wnt/β-catenin signaling to influence the expression of Nanog and Oct4 in gastric cancer cells.

In summary, the mechanism of *H. pylori*-induced CSC-like properties should was examined in this study. As previously described, the *H. pylori*-induced EMT may partly, but not fully, explain this phenotype. Therefore, further exploration of the mechanism of *H. pylori*-induced CSC-like properties is needed. Nanog and Oct4 have the ability to induce CSC-like properties and enhance the EMT, contributing to tumorigenesis [27,38,53,54]. In this study, we demonstrated that CagA-positive *H. pylori* regulates Nanog and Oct4 via Wnt/β-catenin signaling, which promotes the emergence of CSC-like properties in gastric cancer cells. Moreover, we found that CagA-positive *H. pylori* promotes the phosphorylation of β-catenin at Ser675 and Ser552 in a c-met- and/or Akt-dependent manner, which may partly elucidate the mechanisms of the *H. pylori*-induced nuclear accumulation and transcriptional activation of β-catenin. Based on previous reports and our observations, we propose the following model (Fig. 6). First, once CagA is injected into gastric cancer cells via the T4SS during *H. pylori* infection, it directly binds to GSK-3β and depletes its activity, inhibiting β-catenin degradation [12]. Meanwhile, CagA interacts with E-cadherin and disrupts E-cadherin/β-catenin complex formation, releasing in the release of β-catenin from E-cadherin and the unfolding of the protein for Ser675 phosphorylation at the β-catenin C-terminus [11,41]. Then, CagA interacts with c-met and activates the downstream molecule Akt, resulting in phosphorylation of β-catenin at Ser675 and Ser552. Next, this phosphorylation increases nuclear accumulation and transcrip-

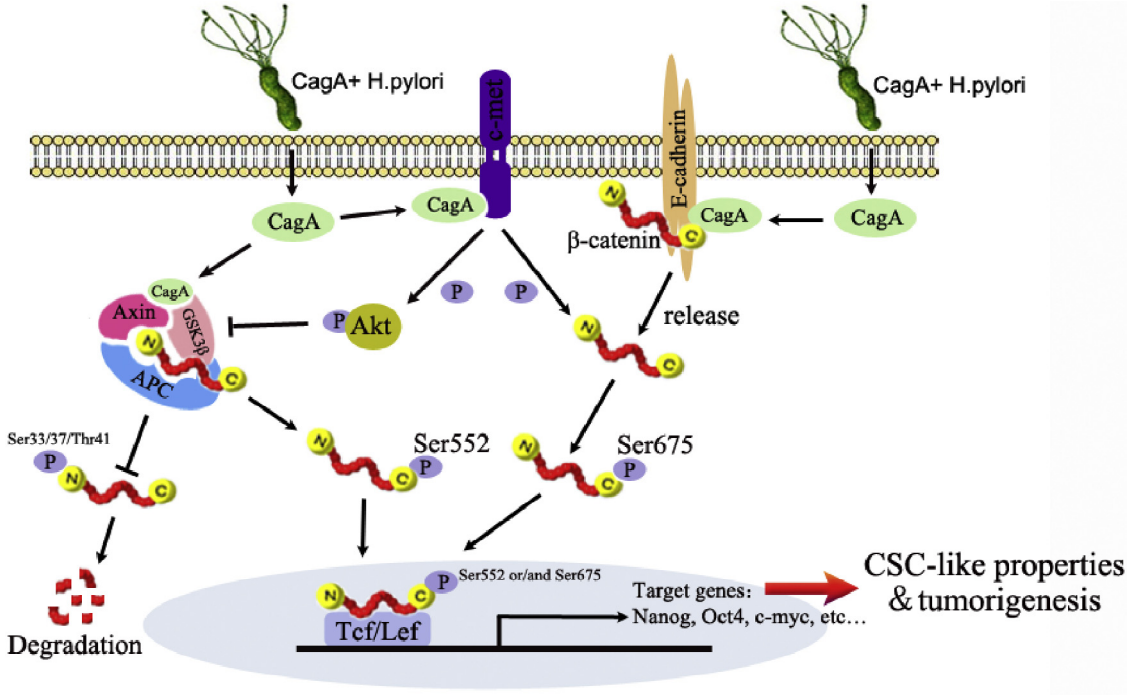

Fig. 6. Model of the *H. pylori*-mediated regulation of Nanog and Oct4 via the Wnt/β-catenin signaling pathway, which promotes cancer stem cell-like properties in human gastric cancer.

tional activity of β-catenin, resulting in increased Wnt/β-catenin signaling. This activation upregulates Nanog and Oct4 expression and promotes the emergence of CSC-like properties in gastric cancer cells.

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Conflict of interest

The authors declare that they have no competing interests.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.02.032.

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