Helicobacter Pylori Induces GATA3-Dependent Chitinase 3 Like 1 (CHI3L1) Upregulation and Contributes to Vascular Endothelial Injuries

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Background: *Helicobacter pylori* infection is associated with various vascular diseases. However, its mechanism is yet to be defined. The present study aimed to investigate the effect of *H. pylori* on vascular endothelial cells as well as the GATA3-related mechanism of *H. pylori* infection-induced endothelial injuries.

Material/Methods: A co-culture of *H. pylori* with human umbilical endothelial cells (HUVECs) was produced. The proliferation of HUVECs that had been incubated with *H. pylori* were examined via CCK-8 (Cell Counting Kit-8) and EdU (5-ethy-nyl-2'-deoxyuridine) staining. Cell migration and microtubules formation were studied using Transwell and tube formation respectively. Construction of a mouse model of *H. pylori* infection as well as the expression of GATA3 and CHI3L1 in vessels were tested using western blot and immunohistochemistry. Small interfering RNA (siRNA) of GATA3 were transfected into HUVECs in order to establish cell lines with knocked-down GATA3. The production of the aforementioned molecules and p38 mitogen-activated protein kinase (MAPK) related molecules in HUVECs was measured using quantitative real-time polymerase chain reaction and western blot.

Results: *H. pylori* significantly inhibited the proliferation, migration, and tube formation of HUVECs, as well as increased the production of the inflammatory factor CHI3L1 and phosphorylated p38 from endothelial cells along with an increased expression of GATA3. Elevated levels of the GATA3 and CHI3L1 were also found in the arteries of *H. pylori*-infected mice. Following GATA3 knockdown, the *H. pylori*-induced dysfunction of HUVECs was restored.

Conclusions: *H. pylori* impaired vascular endothelial function. This might be due to the *H. pylori*-induced increased expression of GATA3, as well as the GATA3 mediated upregulated CHI3L1 and activation of the p38 MAPK pathway.

MeSH Keywords: Cardiovascular Diseases • Chitinase • Endothelial Cells • GATA3 Transcription Factor • *Helicobacter Pylori*

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Background

Helicobacter pylori are gram-negative, spiral shaped bacteria which are considered the most common infectious pathogen of various gastrointestinal disorders, including chronic gastritis, peptic ulcers, gastric cancer, and MALT (mucosa-associated lymphoid tissue) lymphoma [1]. Various studies have found an association of *H. pylori* infection with extra digestive diseases, including neurodegenerative, dermatological, hematologic, cardiovascular, and metabolic diseases [2].

Much existing research has indicated that bacteria including *H. pylori* are closely linked to cardiovascular diseases (CVD) [3,4]. Specific virulence factors, such as cytotoxin-related protein A (CagA) and vacuolating cytotoxin A (VacA), play a leading role in the pathogenesis of *H. pylori* infection [5]. CagA is one of the most intensive virulence factors currently studied, according to whether *H. pylori* strain expresses CagA protein, *H. pylori* is divided into CagA positive (CagA+) strains and (CagA−) negative strains. CagA+ strains can induce more serious gastric mucosal injury and inflammatory reaction than CagA− strains [6]. People with an *H. pylori* infection, especially the CagA+ *H. pylori* infection, are more likely to suffer aortic atherosclerosis and ischemic stroke than those without an *H. pylori* infection [7,8]. Endothelial dysfunction plays an important role in the pathogenesis of CVD, such as atherosclerosis [9]. *H. pylori* infection has the potential to lead to endothelial dysfunction by stimulating either systemic or local immune responses or a chronic inflammatory state, thus further promoting the development of vascular diseases [10]. Endothelial dysfunction marker endothelin-1 was highly expressed in cardiac syndrome patients with anti-CagA IgG [11]. Moreover, infection with Cag-A positive *H. pylori* strain could provoke increased interleukin (IL)-6 and other inflammatory factors in ischemic heart diseases [12]. Some evidence suggested that the eradication of *H. pylori* could therefore reverse endothelial damage and hence decrease the risk of cardiovascular events [13].

The zinc-finger transcription factor GATA3 plays a part in stimulating the direction of cell proliferation, growth, and differentiation in many tissues. Our previous study found that the elevated expression of GATA3 in *H. pylori* was associated with human gastric mucosa, which has also been confirmed in typical gastric epithelial cells GES-1 and Mongolian gerbil animal models with *H. pylori* infection [14]. GATA3 has been found in human coronary artery endothelial cells [15]. Recent research suggests that GATA3-positive macrophages adversely affect ischemia via regulating proinflammatory monocytes/macrophages [16]. Decreases in dietary cholesterol induced regression of atherosclerosis through reduction in Th2 responses, shown as decreased GATA3 levels and that GATA3 can downregulate vascular endothelial growth factor (VEGF) and contribute to regulate endothelial cells inhibited function by regulating

certain microRNA or transforming growth factor(TGF)-β signaling pathways [17,18].

As a common cardiovascular disease, atherosclerosis is increasingly being considered as a chronic inflammatory process, while endothelial cell injury and dysfunction are closely related to inflammatory response [19]. Chitinase 3 like 1 (CHI3L1) can be secreted by vascular endothelial cells, macrophage, and so on [20]. Additionally, it may contribute to the formation of atherosclerosis by impacting vascular endothelial cells and tissue remodeling [21]. Clinical patients and the mouse model serum of CagA+ *H. pylori* infected by atherosclerosis have demonstrated an overexpression of CHI3L1, which is associated with both plaque instability and clinical symptoms [22]. Moreover, it is reported that CHI3L1 reduced cells viability via p38 mitogen-activated protein kinase (MAPK) and PI3K pathways. Enhanced activation of p38 MAPK was involved in regulating cellular responses such as cell migration, survival and apoptosis to various stimuli [23–25].

In this study, we constructed a *H. pylori* and human umbilical vein endothelial cells (HUVECs) co-culture system to investigate whether *H. pylori* infection causes an abnormal function of vascular endothelial cells. We then investigated the GATA3-associated molecular changes in *H. pylori* infected mice model, and related mechanism of *H. pylori*-induced vascular injury *in vitro*.

Material and Methods

Bacterial strains and culture

CagA-positive (CagA+) *H. pylori* and CagA-negative (CagA−) *H. pylori* were respectively isolated from the specimens of gastric ulcer patients under gastroscopy previously. They were then cultivated in microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37°C on Columbia agar plates which contained 10% sheep blood (Bianzhen Biotech, China) and were supplemented with antibiotics (10 mg/L vancomycin, 5 mg/L cefsulodin, 5 mg/L amphotericin, and 5 mg/L trimethoprim) for 3 to 4 days. Following this, the *H. pylori*, which was in good motility and activity for subculture or intervention, was harvested and resuspended in phosphate-buffered saline (PBS, Hyclone, USA). The *H. pylori* concentration was estimated by measuring the $OD_{600\ nm}$, in which $OD_{600\ nm}$ corresponds to approximately 2×10^8 CFU (colony-forming unit)/mL.

Cell culture and treatment

HUVECs were seeded on dishes (Corning, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium (Gibco, USA), supplemented with 10% of fetal bovine serum

(FBS, BI, Israel) and 1% (wt/vol) antibiotic in humidified air with CO_2 5% at 37°C before beginning the experiments. Cultured HUVECs were used for the experiments between passage 19 and 24. *H. pylori* was added to HUVECs at a MOI (multiplicity of infection) of 50, 100, or 150.

Animal model

Male C57BL/6 mice (4-weeks-old) were purchased from the Experimental Animals Department of Third Xiangya Hospital of Central South University in Changsha, China. They were randomly divided into 3 groups: CagA-positive (CagA+) group, CagA-negative (CagA−) group and normal control group (NC). All mice were cultured with the same food and sterile water. After fasting for 24 hours, mice were intragastrically administrated with the CagA+ *H. pylori*, CagA- *H. pylori*, and PBS, respectively. Mice were continuously gavaged for 3 days, then stopped for 1 day. This process was repeated 3 times. Mice were euthanized 4 weeks after the end of the gavage. Obtained gastric tissue underwent a rapid urease test (RUT) as well as a pathological biopsy for detecting *H. pylori* infection. A portion of the thoracic aortic tissue was then gathered and stored in 4% paraformaldehyde, while the other portion was immediately maintained in liquid nitrogen for further histological testing. The medical ethics for this study were approved by the IRB of the Third Xiangya Hospital of Central South University.

Cell proliferation of Cell Counting Kit-8 (CCK-8)

The cell proliferation was firstly detected through a Cell Counting Kit-8 (CCK-8; Vazyme Biotech, China). Briefly, the logarithmic growth phase HUVECs were seeded in 96-well plates, with 2×10^3 cells per well. After 6 hours, the medium which replaced *H. pylori* was added at a MOI of 0, 50, 100, or 150, respectively. Cells were measured at 0, 24, 48, 72, and 96 hours, at which point the supernatant of each well was removed, and cells were incubated in 100 uL DMEM/F12 medium containing 10 uL CCK-8, following the manufacturer's instructions. After incubation for 2 hours at 37°C, the optical density value for each well was measured at 450 nm, using an automated microplate reader.

EdU staining

We used Cell LightTM EdU kit (Ribobio, China) in our research. HUVECs were seeded in 96-well plates at 1×10^4 cells per well. After 6 hours of culture, the cells were replaced with the medium added CagA+ *H. pylori* (MOI=150), CagA− *H. pylori* (MOI=150) and normal medium. After 48 hours, the diluted 100 uL 50 uM EdU (5-ethynyl-2'-deoxyuridine) medium was added to each well for 2 hours. The cells were fixed with 4% paraformaldehyde for 30 minutes, incubated with 2 mg/mL glycine, and shaken for 5 minutes. We then added 0.5%

TritonX-100 PBS for 10 minutes and added 100 uL DAPI (4',6-di-amidino-2-phenylindole), per well, which was incubated in the dark and shaken for 30 minutes before discarding the staining reaction solution. Cells were then PBS-washed, at which point we collected the images using an inverted fluorescence microscope.

Transwell

Cell migration assays were undertaken using a 24-well Transwell chamber (8-μm pore size, Corning, USA). Briefly, after being cultured in the CagA+ *H. pylori* (MOI=150) medium, CagA− *H. pylori* (MOI=150) medium and normal medium for 48 hours, 4×10^5 HUVECs were harvested and resuspended in 100 uL of serum-free medium and added to the top chamber, while DMEM/F12 medium containing 10% FBS was added to complete the bottom room. After incubation at 37°C for 5 hours, cells which adhered to the lower surface were subjected to crystal violet staining and counted under the microscope in 5 predetermined areas.

Tube formation

A Matrigel matrix assay (Corning, USA) was diluted using pre-cooled, serum-free medium, 50 uL Matrigel solution was added to 96-well plates (which were always kept on ice), and the plates were incubated at 37°C for 1 hour to form a gel. After 48 hours, HUVECs cultured in the CagA+ *H. pylori*, CagA− *H. pylori* and normal medium were harvested, and the cells were resuspended in the new normal medium (1×10^5 cells/mL). Following this, 100 uL of cells were seeded in prepared 96-well plates. After 5 hours, tube formation images were captured by microscope. Statistical analysis of the images at 6 hours was performed by ImageJ.

Small interfering RNA (siRNA) construction and transfection

The siRNA against GATA3 and negative control siRNA were constructed and obtained from Invitrogen (GenePharma, China). RNA oligo is shown as follows:
GATA3: sense (5'-3'): GGCUCUACUACAAGCUUCATT,
and antisense (5'-3'): UGAAGCUUGUAGUAGAGCCTT;
Negative control: sense (5'-3'): UUCUCCGAACGUGUCACGUTT;
and antisense (5'-3'): ACGUGACACGUUCGGAGAATT.
HUVECs were then incubated in medium with a mixture of 5 nM siRNA and 12 uL HiperFect Transfection Reagent (Qiagen, USA) in 6-well plates with 3×10^5 cells per well for 24 hours before either adding *H. pylori* or not.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The expressions of GATA3 and CHI3L1 genes mRNA were analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). Briefly, the HUVECs' total RNA was extracted using

E.Z.N.A. Total RNA Kit (OMEGA Bio-Tek, USA), respectively, and the aforementioned samples total RNA was reversely transcribed into cDNA using the RNA reverse transcription kit (TOYOBO, Japan). RT-PCR was then carried out by KOD SYBR qPCR Mix kit (TOYOBO, Japan) and targeting primers. The reaction system was utilized for 20 uL, with reaction conditions of 98°C (2 minutes), 40 cycles of 98°C (10 seconds), 60°C (10 seconds) and 68°C (30 seconds). The melting curve was then analyzed. Experimental apparatus used LightCycler 480 II real-time fluorescent qPCR. The qRT-PCR data were quantified using $2^{-\Delta\Delta Ct}$. All gene primer sequences are shown as follows: GATA3 (human): forward primers (5'-3'): GGAGTGTGTGAACTGTGGGG and reverse primers (3'-5'): TTCGCTTGGGCTTAATGAGG; CHI3L1 (human): forward primers (5'-3'): AAGCAACGATCACATCGACAC and reverse primers (3'-5'): TCAGGTTGGGGTTCCTGTTCT; GAPDH (human): forward primers (5'-3'): GAGCCACATCGCTCAGACAC, and reverse primers (3'-5'): CATGTAGTTGAGGTCAATGAAG.

Western blot

GATA3 and CHI3L1-related protein were both detected using western blot. Following the manufacturer's instructions, the protein of animal arterial tissues or HUVECs were quantitatively analyzed using bicinchoninic acid (BCA) protein concentration determination kit (KeyGEN BioTECH, China). The protein lysate was mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (5×) protein sample buffer (Biosharp, China) then boiled for 5 minutes. The total protein was separated using 10% SDS-PAGE and transferred onto the polyvinylidene difluoride (PVDF) membrane. This membrane was blocked with TBS-T containing 5% low fat dried milk for 2 hours at room temperature. It was then incubated using the primary antibodies of anti-GATA3 rabbit polyclonal antibody (1: 1000, Cusabio, China), the anti-CHI3L1 rabbit polyclonal antibody (1: 600, Cusabio, China), the anti-p38 rabbit polyclonal MAPK antibody (1: 1000, Cell Signaling Technology, USA), the anti-p38 rabbit polyclonal phospho-p38 MAPK antibody (1: 1000, Cell Signaling Technology, USA), the anti-calnexin rabbit polyclonal antibody (1: 1000, Cell Signaling Technology, USA) and the anti-β-actin rabbit polyclonal antibody (1: 1000, Cell Signaling Technology, USA) at 4°C with gentle shaking, overnight. Membranes were later incubated using horseradish peroxidase-conjugated secondary antibodies (1: 10 000, Wuhan Boster Biological Technology, China) at room temperature for 1 hour. An enhanced chemiluminescence (ECL) western blotting detection kit (Advansta, USA) was added to access the images on the chemiluminescent imager, and then ImageJ was used to analyze the gray value of the images.

Immunohistochemical staining

After the tissue was embedded in paraffin, the paraffin section was 4-um thick. The sections were then calcined at 65°C for 60 minutes, dewaxed in xylene and hydrated in gradually diluted ethanol. They were then microwaved in 0.1 M citrate solution high temperature antigen retrieval (pH 6.0) for 10 minutes; after sectioning, slices were incubated with 3% H_2O_2 for 20 minutes at room temperature, before being incubated with goat serum for 20 minutes at room temperature and then incubated with anti-GATA3 rabbit polyclonal antibody (1: 200, Cusabio, China) and anti-CHI3L1 mouse polyclonal antibody (1: 100, Cusabio, China) at 4°C under humid conditions overnight. The next day, the slices were reheated and incubated with the second anti-rabbit/mouse antibody for 20 minutes at room temperature, after which they were washed with PBS and DAB staining (ZSGB-BIO, China) performed. Hematoxylin was then counterstained and was then examined with a microscope. Statistical analysis of the images was conducted using ImageJ.

Statistical analysis

Each experiment was undertaken at least 3 separate times. One-way analysis of variance, 2-way analysis of variance and Student's *t*-test (SPSS 23.0) were used to compare group means. If the 2-tailed *P* value was <0.05 (*), <0.01 (**), <0.001 (***) or <0.0001 (****), it was considered that the data were significantly different.

Results

Inhibition of biological functions of HUVECs after being co-cultured with *H. pylori*

In order to elucidate the direct effects of *H. pylori* on endothelial cells, we selected both CagA-positive (CagA⁺) and CagA-negative (CagA⁻) *H. pylori* strains so that we could co-culture with HUVECs and hence conduct the functional studies. From the CCK-8 assay, we also added *H. pylori* of different concentration gradients (MOI=50, 100, 150) into the medium. When compared with the normal control group (NC), both the CagA⁺ and CagA⁻ *H. pylori* groups showed a relatively poor proliferation ability of HUVECs (Figure 1A). The inhibition effect became more obvious with the passage of time. In contrast, we found that different concentrations of *H. pylori* resulted in differing impacts on cell proliferation. As the concentration of *H. pylori* increased, its effect of inhibiting proliferation was enhanced; when MOI was 150, its effect was statistically significant. The proliferative ability of HUVECs was inhibited after adding *H. pylori* and was presented in a time- and dose-dependent manner. We then selected the shortest beginning

time to exert effects and concentration nodes with relatively noticeable effects and so undertake the following related study. Another method of the EdU staining was used to detect cell proliferation ability. After co-culturing HUVECs and *H. pylori* with MOI=150 for 48 hours, the results were similar to those obtained by the CCK-8 test. In contrast, the CagA+ group leaned more than the CagA− group, although both of these strains inhibited the growth of HUVECs from the EdU test (Figure 1B).

Cell migration was detected by Transwell assays to determine whether *H. pylori* was involved in the movement of HUVECs. It was found that the number of migrated cells in both the CagA+ and CagA- groups was lower than that of NC group. In addition, the CagA+ group presented a lower migration rate than the CagA− group (Figure 1C).

Further, the effects of *H. pylori* on the tube formation ability of HUVECs was conducted. Capillary tube formation was significantly decreased when *H. pylori* were added with MOI=150 in the culture medium when compared to the NC group. Results showed that *H. pylori* could inhibit tube junctions, segments and length (Figure 1D). In line with the previously mentioned experiments, the CagA+ group was found to be more effective than the CagA− group, and was statistically significant.

These results suggested that *H. pylori* has a strong ability to inhibit HUVECs proliferation, migration, and tube formation. Our study demonstrated the direct effect of *H. pylori* on vascular injury at a cellular level, indicating that this process might be involved in the development of vascular diseases.

Increased expression of GATA3 and CHI3L1 in HUVECs with co-cultured *H. pylori*

In order to explore the molecular mechanism of the *H. pylori*-induced endothelial damage, we extracted total RNA from HUVECs using co-cultured CagA+ *H. pylori* or CagA− *H. pylori* and detected the mRNA expression of GATA3. It was found that after the intervention of *H. pylori*, the expression of GATA3 was significantly upregulated. Then we found that the CHI3L1, a molecule associated with inflammation, might be a potential downstream molecule regulated by GATA3, which was predicted by bioinformatics analysis (*http://jaspar2016.genereg.net/*). Interesting, further results showed that the CHI3L1 mRNA increased correspondingly. These changes were more robust in the CagA+ group than in the CagA− group (Figure 2A). We next obtained the same results using western blot (Figure 2B, 2C).

Increased expression of GATA3 and CHI3L1 in *H. pylori* infected mice aorta

We further verified this phenomenon *in vivo*, a CagA+ and CagA− *H. pylori* infected mice model was carried out. GATA3 was

upregulated in *H. pylori*-infected gastric cancer tissues, as demonstrated by our previous study. We therefore initially detected GATA3 and inflammatory molecule CHI3L1 in mice thoracic aorta tissue using immunohistochemical staining. GATA3 and CHI3L1 were shown to be strongly upregulated in vascular endothelial of the thoracic aorta of both CagA+ and CagA− *H. pylori*-infected mice (indicated by the arrow), when compared with normal control mice (Figure 3A, 3B). We then performed western blot on the aortic tissue protein in order to verify these phenomena. In this experiment, there was also an increase in both *H. pylori* groups, and the CagA+ group was found to be more statistically significant (Figure 3C, 3D).

H. pylori induced GATA3 regulates the expression of CHI3L1

We then went on to further explore whether *H. pylori*-induced GATA3 was involved in the regulation of CHI3L1 expression and *in vitro*. Si-RNA was used to knockdown GATA3 in HUVECs. After siRNA-control and siRNA-GATA3 that was transfected, we replaced the medium either with or without *H. pylori* for another 48 hours. The transfection of siRNA-control not obviously affected the expression of GATA3 and other molecules (data not shown). As compared with siRNA-control transfected (si-control) groups, the expression of GATA3 was effectively knocked down in siRNA-GATA3 transfected (si-GATA3) groups (Figure 4A, 4B), and there was no significant difference in GATA3 expression among the groups either with or without *H. pylori* intervention. The expression of CHI3L1 was reduced correspondingly in GATA3 knocked-down cells with the addition of *H. pylori*, while the change was not significant in si-control group (Figure 4A, 4C). These results suggest that GATA3-mediated up-regulated CHI3L1 may be an important mechanism of *H. pylori*-induced endothelia cells injury.

H. pylori mediated GATA3 enhanced the p38 MAPK activation

To further identify signaling pathway involved in *H. pylori* induced vascular endothelial injury, we found that *H. pylori* increased the phosphorylated p38 (p-p38) in HUVECs, consistently, siRNA-control addition made no significant difference in p-p38 expression (data not shown). Moreover, blocking of GATA3 attenuated the *H. pylori* enhanced effect of p-p38 in these endothelial cells as detected by western bot ((Figure 4D).

H. pylori induced GATA3 contributes to the endothelial dysfunction

We then questioned whether GATA3 knockdown could counteract or alleviate the functional abnormality induced by *H. pylori*. The style of HUVECs treatment was the same as before, but only CagA+ *H. pylori* was selected as the representative for
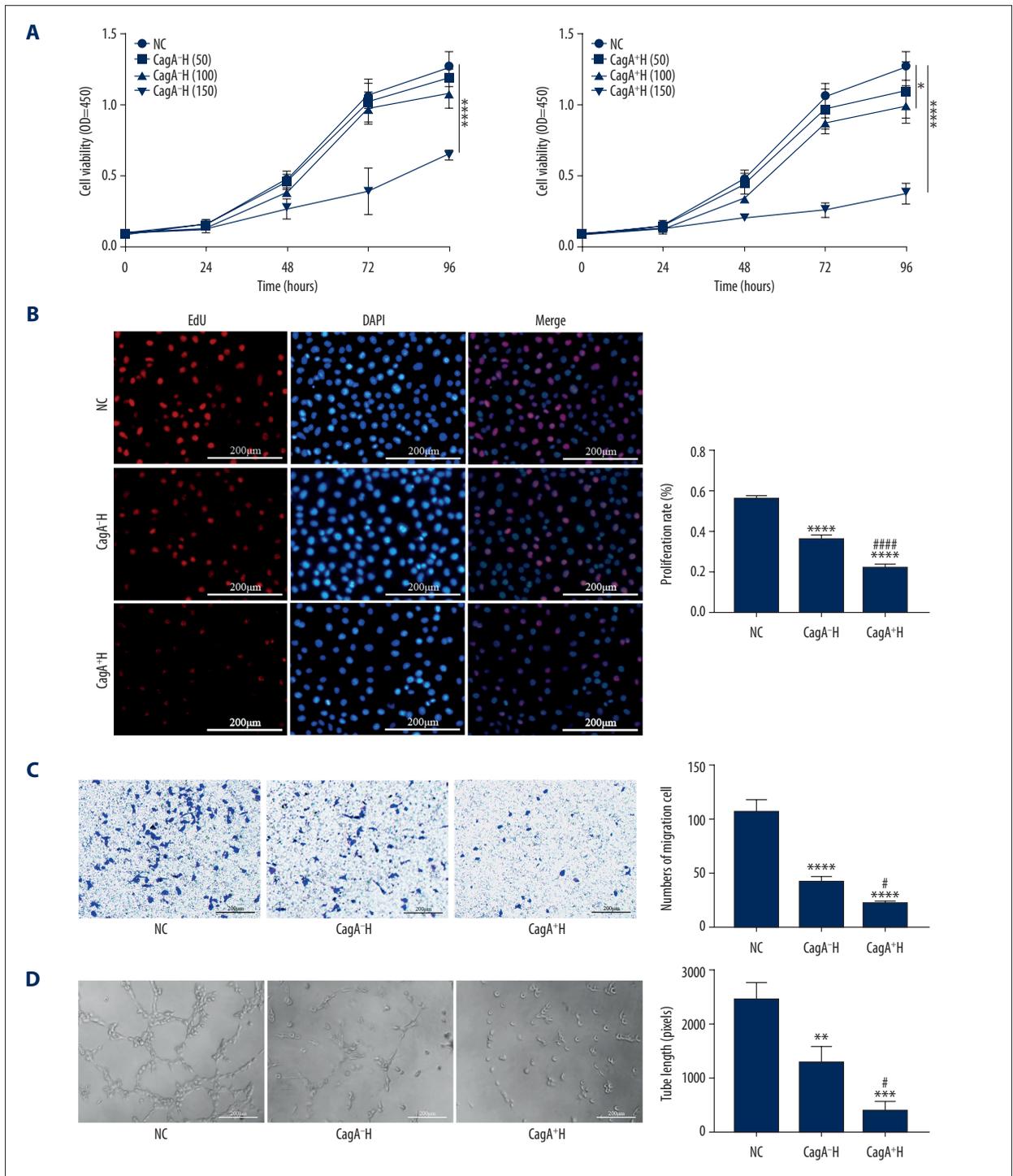
Figure 1. *Helicobacter pylori* inhibited the biological functions of HUVECs. (**A**) CCK-8 assay was used to evaluate the proliferation of the HUVECs after being co-cultured with the CagA⁻ *H. pylori* or CagA⁺ *H. pylori* at the MOI=50, 100, 150, or no *H. pylori*. (**B**) EdU experiments were used to further verify the proliferation of HUVECs inhibited after being co-cultured with the CagA⁻ *H. pylori* or CagA⁺ *H. pylori* at MOI=150 for 48 hours. (**C**) Transwell experiments demonstrated the migration function of HUVECs was declined after being co-cultured with *H. pylori*. (**D**) Tube formation assays showed the ability of forming tubular structures of HUVECs was decreased after being co-cultured with *H. pylori*. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ versus NC group. # $P<0.05$, #### $P<0.0001$ versus CagA⁻ group. HUVECs, human umbilical endothelial cells; CCK-8 – Cell Counting Kit-8; CagA – cytotoxin-related protein; MOI – multiplicity of infection; EdU – 5-ethynyl-2'-deoxyuridine.
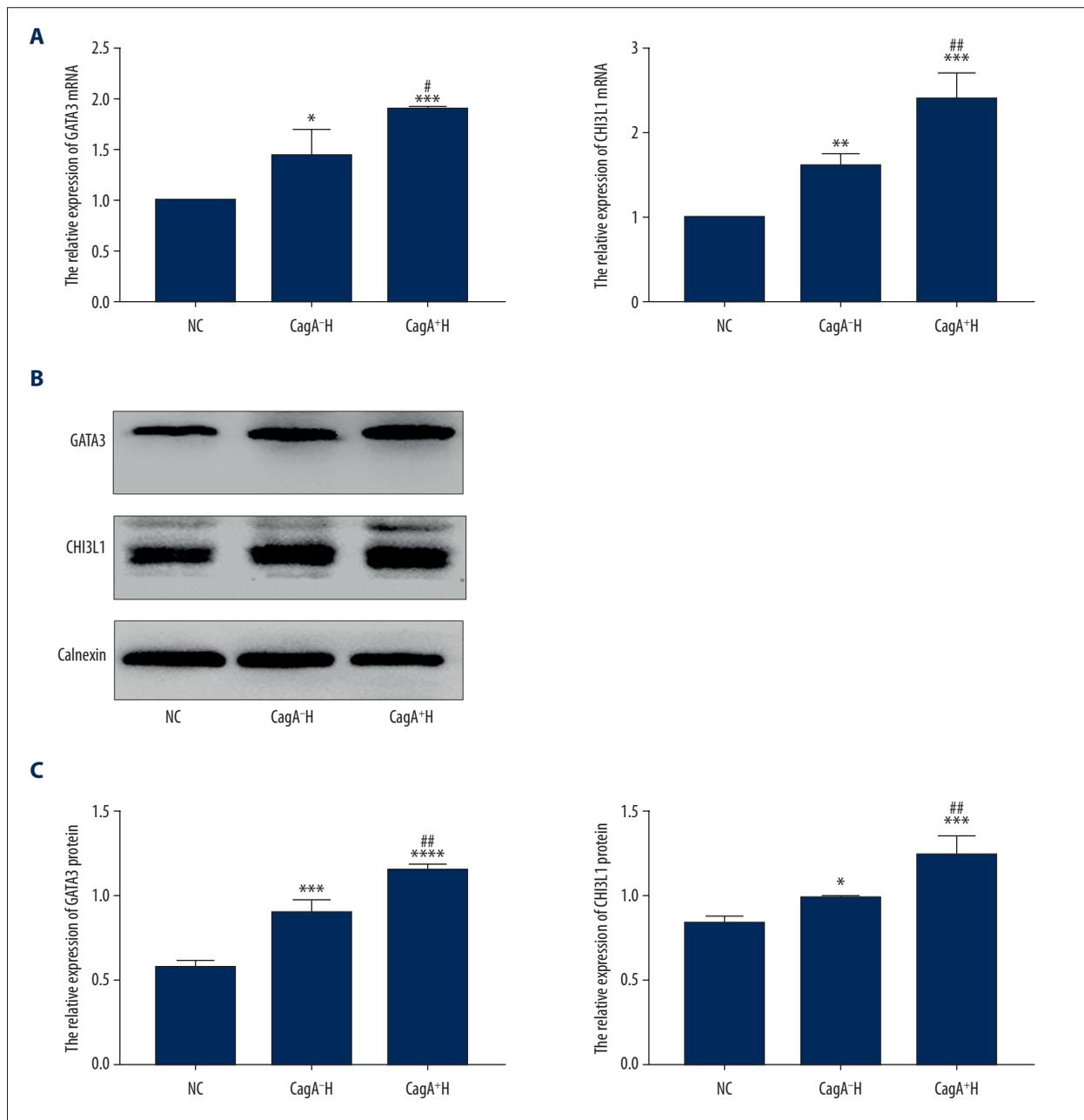
Figure 2. The expression of GATA3 and CHI3L1 were upregulated in HUVECs co-cultured with *Helicobacter pylori*. (**A**) qRT-PCR was used to show the expression of GATA3 and CHI3LI mRNA in HUVECs after being cultured with medium added with CagA⁻ *H. pylori* or CagA⁺ *H. pylori* or PBS for 48 hours. (**B, C**) Western blot was used to show the elevated expression of GATA3 and CHI3L1 protein of the aforementioned cells, Calnexin levels were used as a loading control. * $P<0.05$, ** $P<0.01$, *** $P<0.0001$ **** $P<0.0001$ versus NC group. # $P<0.05$, ## $P<0.01$ versus CagA⁻ group. HUVECs – human umbilical endothelial cells; CagA – cytotoxin-related protein; qRT-PCR – quantitative real-time polymerase chain reaction; PBS – phosphate-buffered saline.
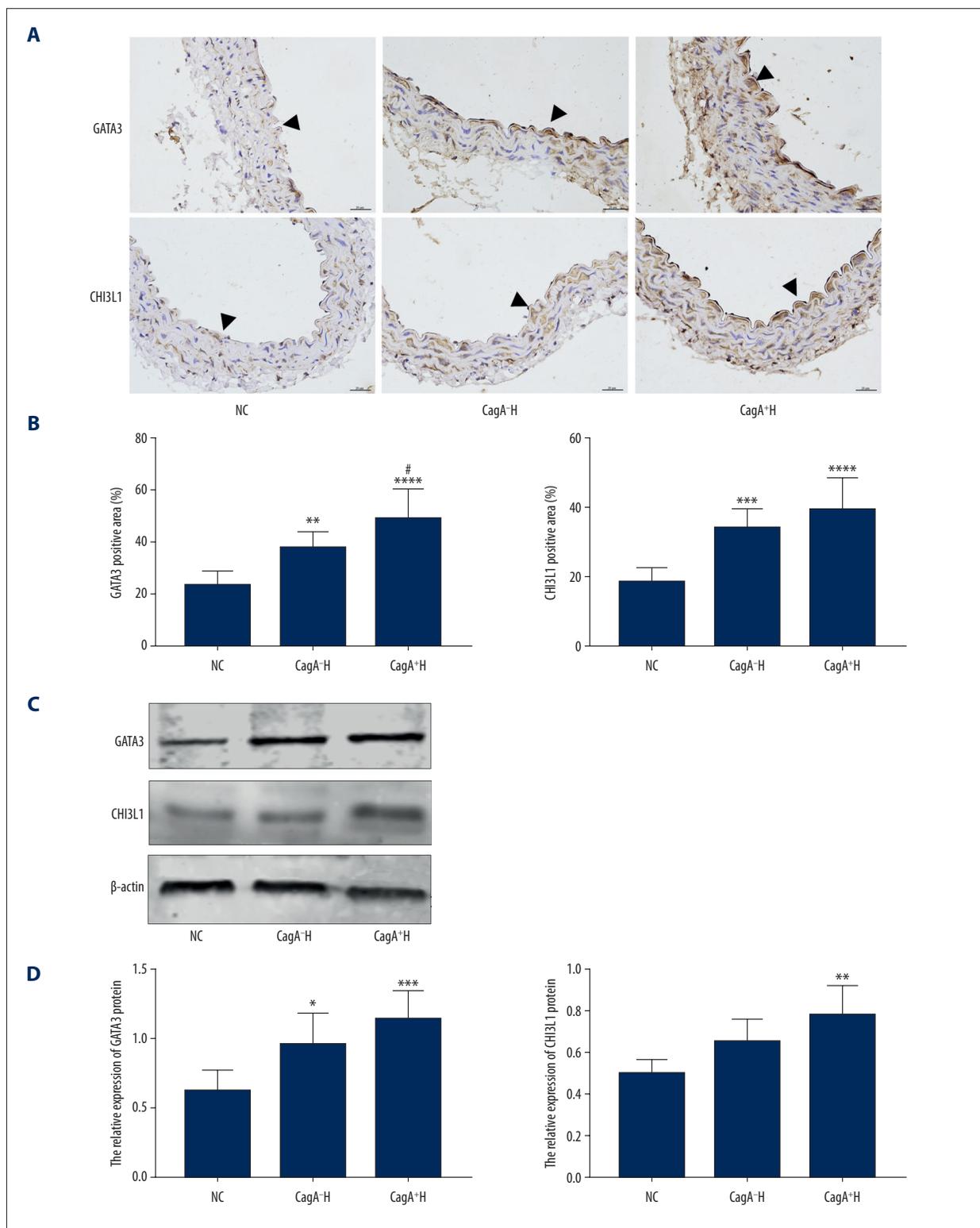
Figure 3. The expression of GATA3 and CHI3L1 were elevated in the *Helicobacter pylori* infected mice model. (**A, B**) Immunohistochemical staining was used to demonstrate the upregulation of GATA3 and CHI3L1 in mice thoracic aorta after being gavaged with CagA+ *H. pylori* or CagA− *H. pylori*. (**C, D**) Western blot used to show the expression of GATA3 and CHI3L1 protein of the aforementioned mice thoracic aorta, β-asctin levels were used as a loading control. * *P*<0.05, ** *P*<0.01, *** *P*<0.0001 **** *P*<0.0001 versus NC group. # *P*<0.05 versus CagA− group. CagA – cytotoxin-related protein.
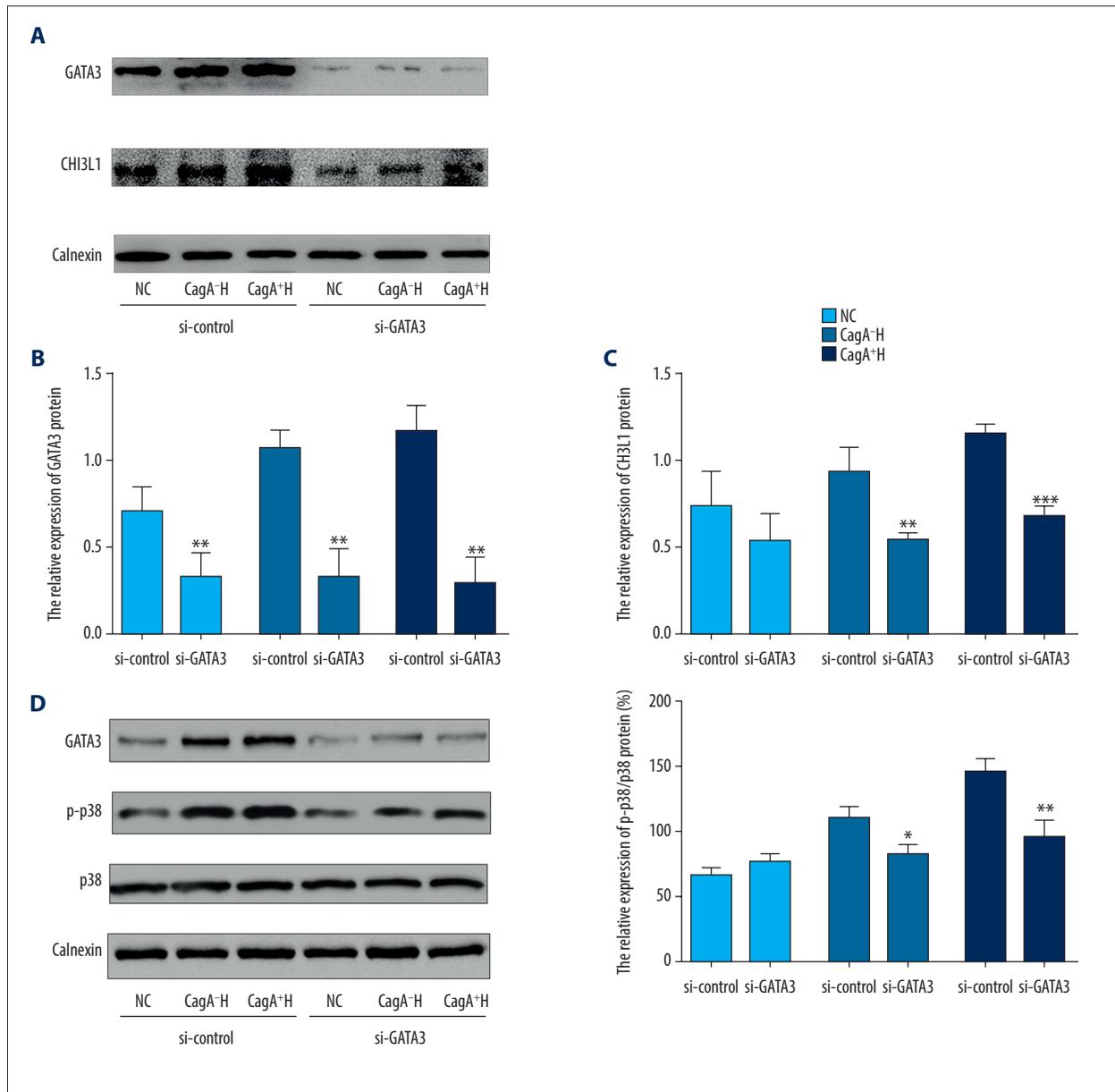
Figure 4. Small interfering RNA (siRNA)-mediated knockdown of GATA3 lowered *Helicobacter pylori* -induced expression of CHI3L1 and p38 phosphorylation (**A**) Western blot shows the expression of GATA3 and CHI3L1 protein in HUVECs after transfected with siRNA-control or siRNA-GATA3 for 24 hours and cultured with medium added with CagA⁻ *H. pylori* or CagA⁺ *H. pylori* or PBS for 48 hours. Calnexin levels were used as a loading control. (**B**) The expression of GATA3 was significant reduced by siRNA-GATA3 transfected. (**C**) The elevated expression of CHI3L1 caused by *H. pylori* was relatively decreased as GATA3 was knocked down. (**D**) The *H. pylori*-induced activation of p38 MAPK was attenuated in GATA3 knocked-down cells. * *P*<0.05, ** *P*<0.01, *** *P*<0.0001 versus si-control group. HUVECs – human umbilical endothelial cells; CagA – cytotoxin-related protein; PBS – phosphate-buffered saline MAPK – mitogen-activated protein kinase.
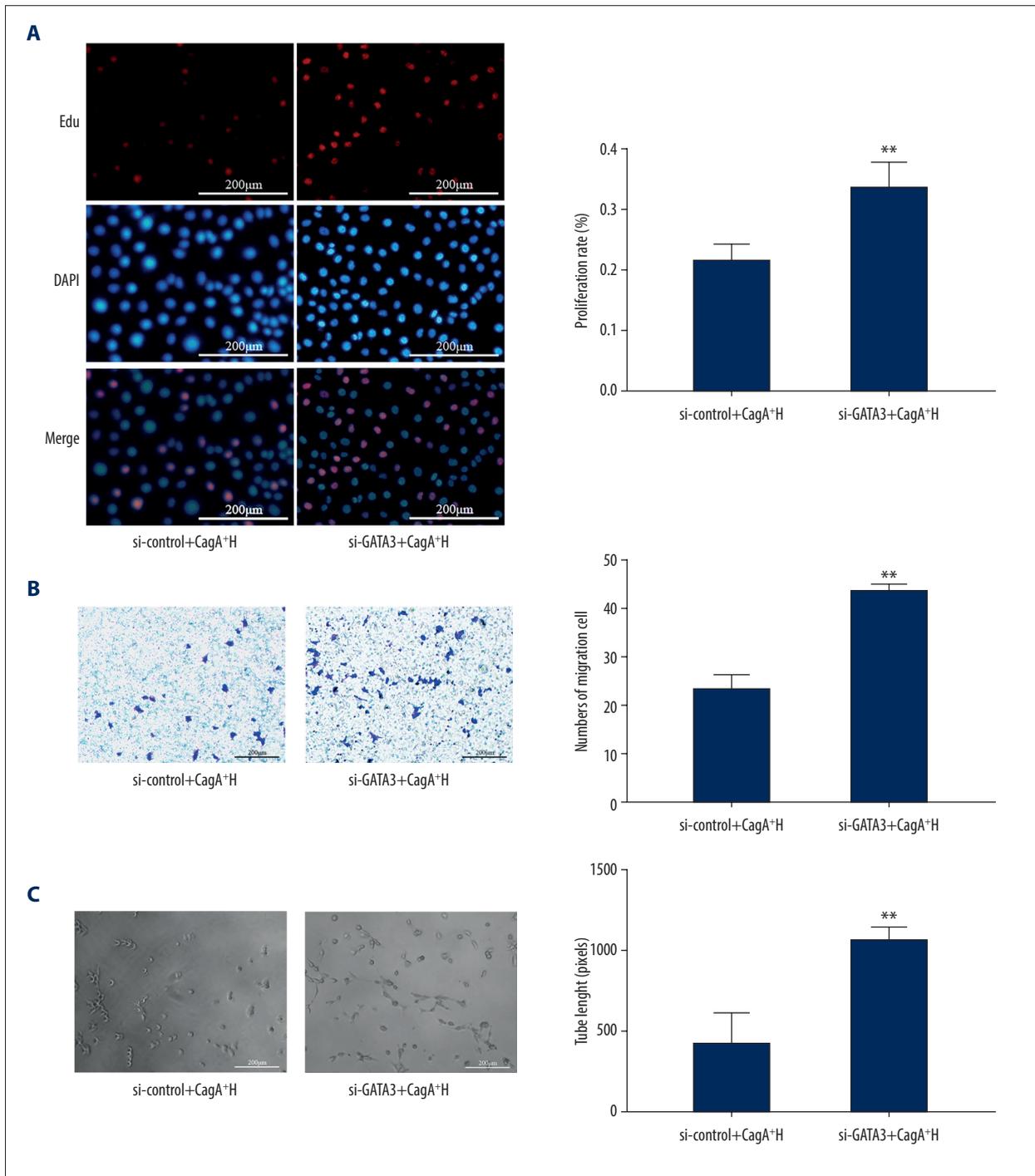
Figure 5. Knockdown of GATA3 restored the CagA+ *Helicobacter pylori*-induced inhibitory function in HUVECs. (**A**) The EdU staining showed the inhibited proliferation by CagA+ *H. pylori* was restored after GATA3 knockdown. (**B**) Transwell experiments demonstrated the migration function was increased after GATA3 knockdown. (**C**) Tube formation ability was partly repaired after GATA3 knockdown. ** $P<0.01$ versus si-control+CagA+ H group. CagA – cytotoxin-related protein; HUVECs – human umbilical endothelial cells; EdU – 5-ethynyl-2'-deoxyuridine.

comparison. EdU staining showed the inhibitory proliferation of HUVECs induced by *H. pylori* was elevated due to GATA3 knockdown (Figure 5A). In addition, Transwell assays demonstrated the enhanced migratory potential, while tube formation suggested a relatively functional recovery (Figure 5B, 5C).

Discussion

H. pylori infection has a rate of approximately 50% of the human population, with a higher infection rate found in developing countries [26]. Cardiovascular disease (CVD) remains a high burden worldwide, and the prevalence of CVD will continue to grow as the global population ages, increasing cardiovascular risk factors in countries where the economy is still developing [27]. Thus, we have focused on the relationship between *H. pylori* infection and cardiovascular diseases as well as its underlying mechanism.

It is a common knowledge that *H. pylori* colonizes in gastric mucosa. However, previous research has suggested that *H. pylori*-specific DNA was detected in the aorta of patients who had coronary artery and carotid artery diseases, and that *H. pylori* urea was found in human atherosclerotic plaque [28–30]. In other words, these research studies suggested that some components of *H. pylori* had the ability to reach the blood vessels, while vascular endothelial cells are the first to be exposed to the *H. pylori* composition. We therefore used a co-culture of *H. pylori* and vascular endothelial cells (HUVECs) to conduct a preliminary exploration of its possible direct damage to blood vessels.

Previous studies have demonstrated the antiproliferative effect of *H. pylori* on endothelial cells [31]. The results of this current study suggest that the proliferation, migration and tube formation of HUVECs are all inhibited by the direct stimulation of *H. pylori*. Dysfunction of vascular endothelial cells might be regarded as the initial step of atherosclerosis as well as other CVDs. Impaired proliferative and migratory capacity increase vascular endothelial permeability [10,32]. Our results suggest that *H. pylori* might be a damaging stimulus which triggers vascular endothelial injury and adversely affects early vascular repair, thereby increasing patients' susceptibility to vascular disease. Interestingly, CagA+ *H. pylori* exhibited stronger virulence when compared with CagA− *H. pylori*, which is consistent with the existing clinical research mentioned [33,34].

We then investigated which elements of the conceivable molecule mechanism *H. pylori* brought to vascular endothelial cells. According to our previous study, an increased expression of GATA3 induced by *H. pylori* might participate in skewed type-2 immune responses [35], which suggested that the increase of GATA3, induced by *H. pylori*, has the potential to effect more than just the stomach. Reduced GATA3 levels of Th2 responses

induced a regression of atherosclerosis and other inflammatory diseases [18,36]. A preceding study demonstrated that GATA-2 and GATA3 were identified in human coronary artery endothelial cells [15]. In this study, *H. pylori*-infected mouse vascular tissue and HUVECs following *H. pylori* intervention both indicated an increased expression of GATA3. We continued to investigate the ways in which GATA3 is involved in vascular injury. Long-term inflammation is a cause of cardiovascular disease, and *H. pylori* infection is a chronic inflammation [37]. Overexpression GATA3 involved in the inflammation of allergic asthma [38]. Recently, CHI3L1 has been indicated as a novel biomarker for inflammation and has been shown to be involved in the pathogenesis of endothelial dysfunction and early onset atherosclerosis [39,40]. CHI3L1 exhibited atherogenic effects by inducing monocyte adhesion and reducing NO generation [41]. Our *in vivo* and *vitro* data showed an increased CHI3L1, which was induced by *H. pylori* invasion. Following knockdown of GATA3 in HUVECs, the endothelial function was improved and CHI3L1 expression of *H. pylori*-induced was attenuated. In addition, the intervention of *H. pylori* markedly increased the expression of p-p38 as well as p-p38/p38. p38 MAPK signaling appears crucial in endothelial inflammation and dysfunction. Previous studies showed that CHI3L1 induced p38 MAPK activation in human monocytic cells, further linking CHI3L1 to coronary artery disease [42]. The current study has showed that the absence of GATA3 could attenuate on *H. pylori*-stimulated p38 phosphorylation and activation. Thus, we infer that the *H. pylori* might aggravate vascular injury and chronic inflammation through a GATA3-mediated elevation of CHI3L1 as well as regulating p38 MAPK pathway.

The data reveal the novel mechanisms for the development of cardiovascular diseases in the patients with *H. pylori* infection, and help exploring new and effective strategies to preventing and treating cardiovascular diseases associated with *H. pylori* infection. However, some limitations of this study should be noted, including the ways in which the components of *H. pylori* reach vascular cells and limited signaling pathways experiments of pathogenic mechanism. In addition, the deeper vascular function research must be further explored *in vivo* must be further explored *in vivo*.

Conclusions

In summary, *H. pylori* significantly induced the dysfunction of vascular endothelial cells with increased expression of GATA3 and CHI3L1. GATA3 knockdown alleviated *H. pylori* – induced functional impairment by regulating CHI3L1 and involved p38 MAPK pathway.

Conflicts of interest

None.

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