Phosphorylation of phosphatase and tensin homolog induced by *Helicobacter pylori* promotes cell invasion by activation of focal adhesion kinase

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Abstract. Phosphorylation of the phosphatase and tensin homolog (PTEN) tumor suppressor at Ser380/Thr382/Thr383 residues is a novel mechanism underlying PTEN inactivation in gastric carcinogenesis, which may be triggered by *Helicobacter pylori* infection. To investigate this further, the effect of *H. pylori* infection on PTEN phosphorylation and the subsequent activation of focal adhesion kinase (FAK), were evaluated in gastric tissue samples from Mongolian gerbils and in the human gastric epithelial mucosa cell line GES-1 using immunohistochemistry, western blotting and Transwell assays. The *in vivo* and *in vitro* results of the present study demonstrated that *H. pylori* infection induced the phosphorylation and inactivation of PTEN at Ser380/Thr382/383, and the subsequent phosphorylation and activation of FAK at Tyr397. Gastric epithelial cell invasion was also increased. Furthermore, stable expression of a dominant-negative PTEN mutant inhibited the enhanced FAK activation and cell invasion induced by *H. pylori* infection. These results suggest that the mechanism underlying *H. pylori*-induced carcinogenesis may involve promoting cell invasion through the phosphorylation of PTEN and the activation of FAK.

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Introduction

Helicobacter pylori is a gram-negative bacterial species that represents the most common bacterial infection of the gastric epithelium worldwide (1,2). *H. pylori* colonization is the most prominent identified risk factor for malignancies that arise within the stomach, and it increases the risk of atrophic gastritis, intestinal metaplasia, and distal gastric adenocarcinoma (3-6). *H. pylori* infection has been classified by the World Health Organization as a carcinogen for the development of gastric carcinoma (7); this cancer type is the second most common cause of cancer-associated mortality worldwide, accounting for ~989,600 new cases and ~738,000 mortalities per year, half of which occur in eastern Asia (8,9).

Since the initial description of *H. pylori* by Marshall and Warren in 1984 (10), significant advances towards understanding gastric carcinogenesis have been achieved. Gastric carcinogenesis is a multifactorial and multistep process that involves the activation of oncogenes and the inactivation of tumor suppressor genes (11-16). One such tumor suppressor gene is phosphatase and tensin homolog (PTEN) (17-19), which has lipid and protein phosphatase activities. PTEN inactivation has been observed in glioblastomas, endometrial carcinomas and skin, prostate and breast cancers (17-19). Previous studies have indicated that PTEN inactivation in gastric cancers may be due to genetic or epigenetic changes, including mutation, loss of heterozygosity, promoter hypermethylation, regulation of microRNA and post-translational phosphorylation (14,20-25). Phosphorylation of PTEN on sites within the C2 domain (Ser380, Thr382 and Thr383) decreases phosphatase activity and increases stability (26,27), which may cause loss of tumor suppressor function and increased susceptibility to cancer. In our previous study, it was demonstrated that PTEN phosphorylation at residues Ser380/Thr382/Thr383 is a novel mechanism underlying PTEN inactivation in gastric carcinogenesis (14,28). This was observed to be triggered by *H. pylori* infection and required for the activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B signaling pathway and the subsequent promotion of cell survival (14).

PTEN regulates a variety of other downstream signaling pathways, including the regulation of cell migration, invasion

and growth by focal adhesion kinase (FAK) (29-32). FAK is a key molecule that is implicated in integrin signaling, which contributes to cancer progression, invasion and metastasis (29,33,34). Previous studies have identified that *H. pylori* may induce FAK activation and cytoskeletal reorganization (35,36). Therefore, the aim of the present study was to determine the effect of *H. pylori*-induced PTEN phosphorylation on the activation of FAK *in vivo* and *in vitro*, in order to identify the mechanisms underlying gastric cancer development.

Materials and methods

H. pylori strain. As previously described (14,28), the CagA[+] and VagA[+] *H. pylori* strain ATCC43504 (National Institute for Communicable Diseases and Prevention of Chinese Center for Disease Control and Prevention, Beijing, China) was cultured on *Campylobacter* agar plates (Shanghai Municipal Center For Disease Control and Prevention, Shanghai, China) supplemented with 10% sheep blood (Shanghai Kangfeng, Biological Technology Co., Ltd, Shanghai, China) and incubated at 37°C under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) for 24 h, then subcultured in Brucella broth (Shanghai Municipal Center For Disease Control and Prevention) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) at 37°C under a microaerophilic atmosphere for 16-18 h. Bacterial density was estimated spectrophotometrically as the absorbance at 600 nm [optical density $(OD)_{600}$], and viable counts were determined as colony-forming units (CFU)/ml (1 OD_{600}=10^9 CFU/ml).

Mongolian gerbils. A total of 79 specific pathogen-free male Mongolian gerbils (age, 5-8 weeks; weight, 30-50 g) purchased from the Zhejiang Academy of Medical Sciences (Hangzhou, China), were maintained in an isolated clean room with regulated temperature (20-22°C), humidity (~55%) and a 12/12-h light/dark cycle with *ad libitum* rodent diet and water. As previously described (14,28), following one week of observation, the gerbils were administered 1 ml orogastric infusions of sterile Brucella broth (n=25; controls) or 1x10^9 CFU of *H. pylori* (n=54) once every three days, for a total of 10 infusions. Gerbils were fasted for 12 h prior to *H. pylori* inoculation and drinking water was withheld following the inoculation. Food and water were freely available to the gerbils at 4 h post-inoculation. The animals were euthanized using sodium pentobarbital (150 mg/kg) and cervical dislocation at 6 months (*H. pylori*-infected, n=30; controls, n=15) or 12 months (*H. pylori*-infected, n=24; controls, n=10). Then gastric tissue was separated from the animals, which were cut along the greater curvature of stomach, the gastric tissue was smoothed out and linear strips of gastric tissue extending from the squamocolumnar junction through the proximal duodenum were collected. Detection of *H. pylori* infection was carried out using Giemsa staining (14). All animal experiments and procedures were approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University (Nanchang, China).

Cell line and lentivirus infection. The GES-1 immortalized human gastric epithelial mucosa cell line from the Beijing Institute for Cancer Research (Beijing, China) was cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, Utah, USA) supplemented with 10% FBS, 100 U penicillin and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) at 37°C in an atmosphere containing 5% CO_2. DMEM supplemented with 10% FBS to suspend the *H. pylori* was added to infect GES-1 cells with various multiplicity of infection (MOI) for 0, 0.5, 1, 3 and 6 h. The same volume of DMEM (5 ml) supplemented with 10% FBS was added to GES-1 cells in the control group.

Wild-type PTEN, dominant-negative mutant PTEN (C124S) and empty lentiviral supernatants were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). For lentiviral infection, GES-1 cells were cultured to ~60% confluence and incubated with the aforementioned three lentiviral supernatants, respectively, and hexadimethrine bromide (Sigma-Aldrich, St. Louis, MO, USA) for 6 h as aforementioned. After 48 h, the cells were split and cultured in selection media supplemented with blasticidin (6 μg/ml; Sigma-Aldrich) for a further 2 weeks at 37°C in an atmosphere containing 5% CO_2. The stable cell lines expressing a wild type or dominant-negative mutant PTEN were then established. As previously described, PTEN was overexpressed in stable cell lines carried with wild-type PTEN (28). Dominant-negative mutant PTEN (C124S) contributed to the phosphorylation and inactivation of PTEN at residues Ser380/Thr382/383. In addition, the empty vector did not contain exogenous gene.

Immunohistochemistry. Immunohistochemistry was performed on paraffin-embedded sections of Mongolian gerbil gastric tissues using anti-PTEN (cat. no. ab31392; dilution 1:150), anti-phosphorylated (p)-PTEN (Ser380/Thr382/Thr383; cat. no. ab47332; 1:800), anti-FAK (cat. no. ab40794; 1:400), and anti-p-FAK (Tyr397; cat. no. ab4803; 1:400) antibodies (all from Abcam, Cambridge, UK) and polyperoxidase-conjugated anti-mouse or rabbit immunoglobulin G antibody as provided in PV-9000 Polymer Detection System (Zhongshan Goldenbridge, Beijing, China) using previously described methods (12,14,15). The stained sections were selected, reviewed and scored from five randomly selected high-power fields (40x objective lens) by two pathologists blinded to the histopathological data. Grading discrepancies were re-reviewed and discussed to obtain a final score. Epithelial cells with yellow or brown staining in the nucleus and/or cytoplasm were defined as positive for immunoreactivity. The percentage of immunoreactive cells from 100 cells selected for each field, were averaged from the five fields and scored as follows: 0, ≤5.0%; 1, 5.1-25.0%; 2, 25.1-50.0%; 3, 50.1-75.0%; and 4, >75.1% immunoreactivity. Furthermore, the staining intensity was also semi-quantitatively assessed as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. The overall protein expression level was presented as a grade calculated from the integrated scores of the 'area x intensity', as follows: Grade 1, score 0-2 (negative); grade 2, score 3-5 (weakly positive); grade 3, score 6-8 (moderately positive); and grade 4, score 9-12 (intensely positive).

Immunoblotting. Western blotting was performed according to standard methods as described previously (13) using
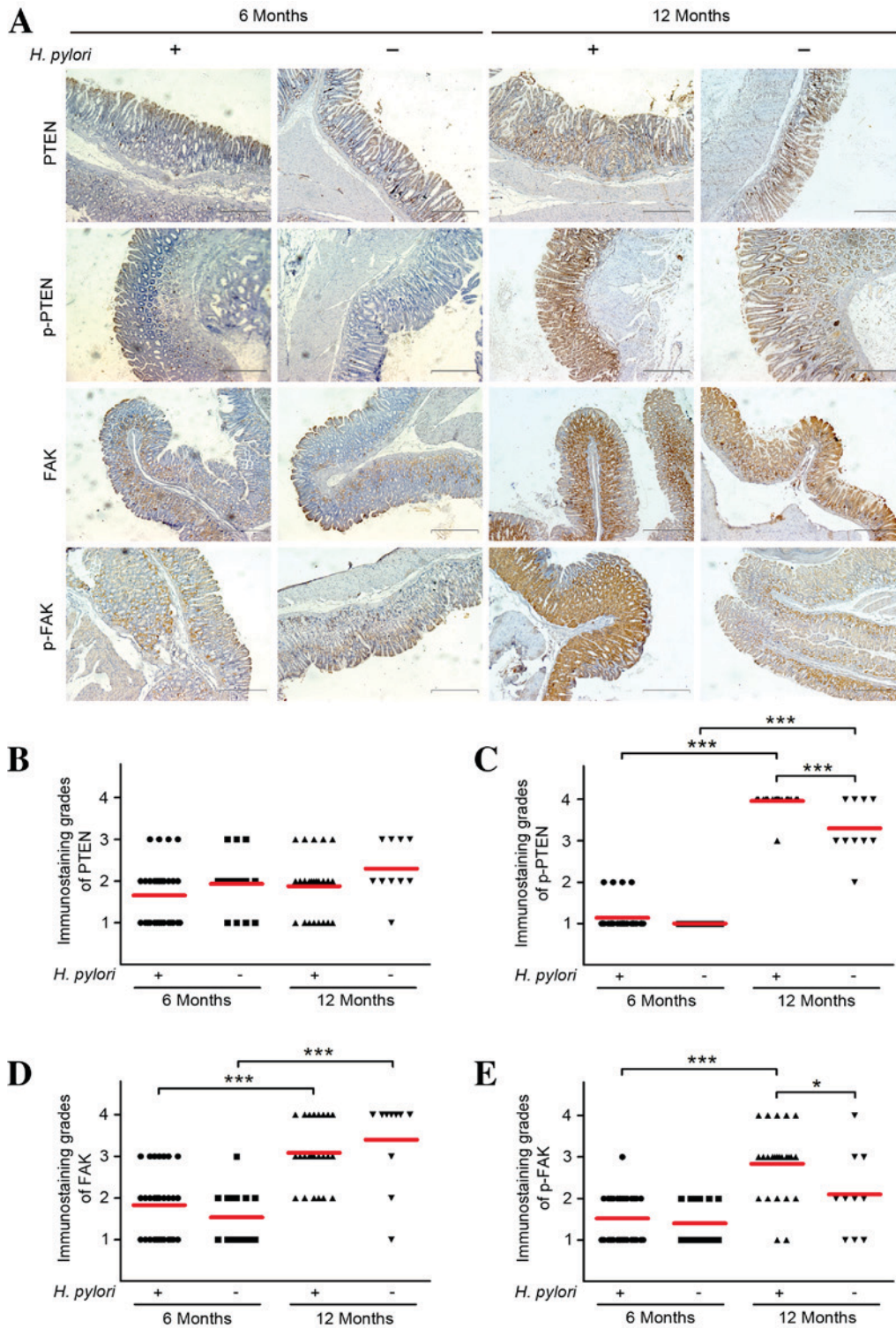
Figure 1. Immunohistochemical staining of PTEN, p-PTEN, FAK and p-FAK in the gastric mucosal regions of Mongolian gerbils. Gastric tissue sections from *H. pylori*-infected gerbils were collected at 6 or 12 months following inoculation and stained using antibodies against PTEN, p-PTEN, FAK and p-FAK. (A) Representative images of immunohistochemical staining are shown (scale bars, 200 μm). Immunoreactivity levels of (B) PTEN, (C) p-PTEN, (D) FAK and (E) p-FAK in the tissues were semi-quantitatively assessed, and the protein expression levels were expressed as grades 1-4. Mean grades (red lines) for each protein in each group are shown. *P<0.05; ***P<0.001. PTEN, phosphatase and tensin homolog; p-, phosphorylated; FAK, focal adhesion kinase; *H. pylori*, *Helicobacter pylori*.

anti-PTEN (cat. no. 9559; Cell Signaling Technology, Inc., Danvers, MA, USA; dilution 1:10,000), anti-p-PTEN (Ser380/Thr382/Thr383; cat. no. 9554; Cell Signaling Technology, Inc.; dilution 1:10,000), anti-FAK (cat. no. ab40794; Abcam; 1:1,000), anti-p-FAK (Tyr397; cat.

no. ab4803; Abcam; 1:1,000) and anti-β-actin (sc-1615-R; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies. Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA; version 4.5.1) was used for densitometric measurements of band intensities.
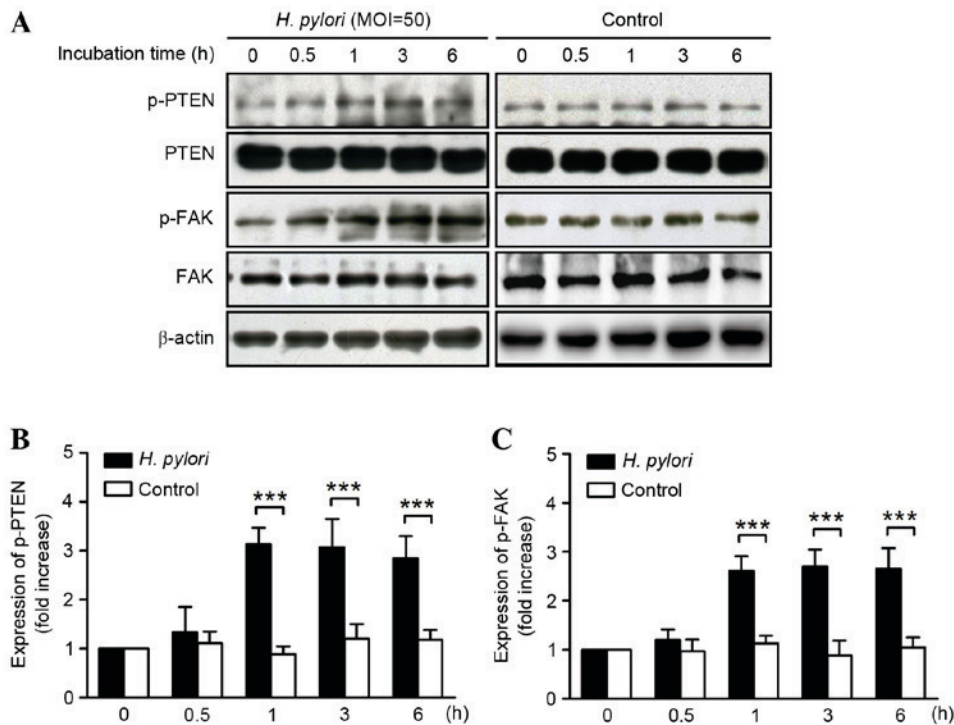
Figure 2. Expression levels and phosphorylation of PTEN and FAK in GES-1 gastric epithelial cells following incubation with *H. pylori*. (A) Representative western blots from GES-1 cell lysates following incubation with *H. pylori* for 0, 0.5, 1, 3 and 6 h (MOI, 50) are shown. Graphs represent quantitative analysis (expressed as the fold of the control) of (B) p-PTEN and (C) p-FAK expression levels; data are presented as the mean ± standard deviation of triplicated experiments. ***P<0.001. PTEN, phosphatase and tensin homolog; p-, phosphorylated; FAK, focal adhesion kinase; *H. pylori*, *Helicobacter pylori*.

Invasion assays. GES-1 cells were seeded at a density of 1×10^4 cells/well in Transwell chambers (8.0 μm pore size; Corning Inc., Corning, NY, USA) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), with medium supplemented with 10% FBS in the lower chamber as the chemoattractant and medium supplemented with 0.2% BSA in the upper chamber, then incubated for 20 h at 37°C with 5% CO_2. Cells that did not migrate through the pores were manually removed with a cotton swab. Cells adhered to the bottom of the chamber membrane were fixed in methanol at room temperature for 5 min and stained with hematoxylin. In three independent experiments, the number of migrated cells on the lower surface of the membrane was counted in 10 visual fields per well using a light microscope at x400 magnification and means of these counts were analyzed for statistical significance.

Statistical analysis. The data are presented as the mean ± standard deviation or the fold of the control. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Student's t-tests or Mann-Whitney U tests were used to compare the differences in the mean value between various groups. P<0.05 was considered to indicate a statistically significant difference.

Results

H. pylori infection increases PTEN and FAK phosphorylation in vivo. The Mongolian gerbils were successfully infected with *H. pylori*, which was confirmed by Giemsa staining. No animals challenged with Brucella broth alone exhibited detectable evidence of *H. pylori* infection.

Immunohistochemical analysis revealed no significant difference in PTEN expression levels from *H. pylori* infection at 6 or 12 months following inoculation (Fig. 1). Inhibitory p-PTEN demonstrated variable protein expression levels in all groups, with the exception of the control group after 6 months. The expression levels of p-PTEN were significantly higher in the gastric mucosae of Mongolian gerbils at 12 months following *H. pylori* infection (P=0.000) compared with the control, but not in the corresponding groups after 6 months. The PTEN expression levels were higher at 12 months than at 6 months with or without *H. pylori* infection. Immunohistochemical analysis of FAK revealed no difference in expression levels from *H. pylori* infection after 6 or 12 months compared with the controls; however, the expression levels were significantly higher at 12 months compared with at 6 months in the groups with or without *H. pylori* infection (P=0.000; Fig. 1). By contrast, after 6 months, active p-FAK expression levels were not observed to be altered by *H. pylori* infection compared with the control group, whereas the expression levels were significantly higher compared with the controls after 12 months (P=0.041). Furthermore, statistical analysis demonstrated that p-FAK expression levels in *H. pylori*-infected gerbils were significantly higher after 12 months compared with 6 months infection (P=0.000).

H. pylori increases PTEN and FAK phosphorylation in vitro. Western blot analysis of protein lysates from GES-1 cells demonstrated that incubation with *H. pylori* increased the phosphorylation of PTEN from 1 h, which was observed to be maintained for ≤6 h (P<0.001) without altering the total PTEN protein expression levels (Fig. 2A). Relative to 0 h,
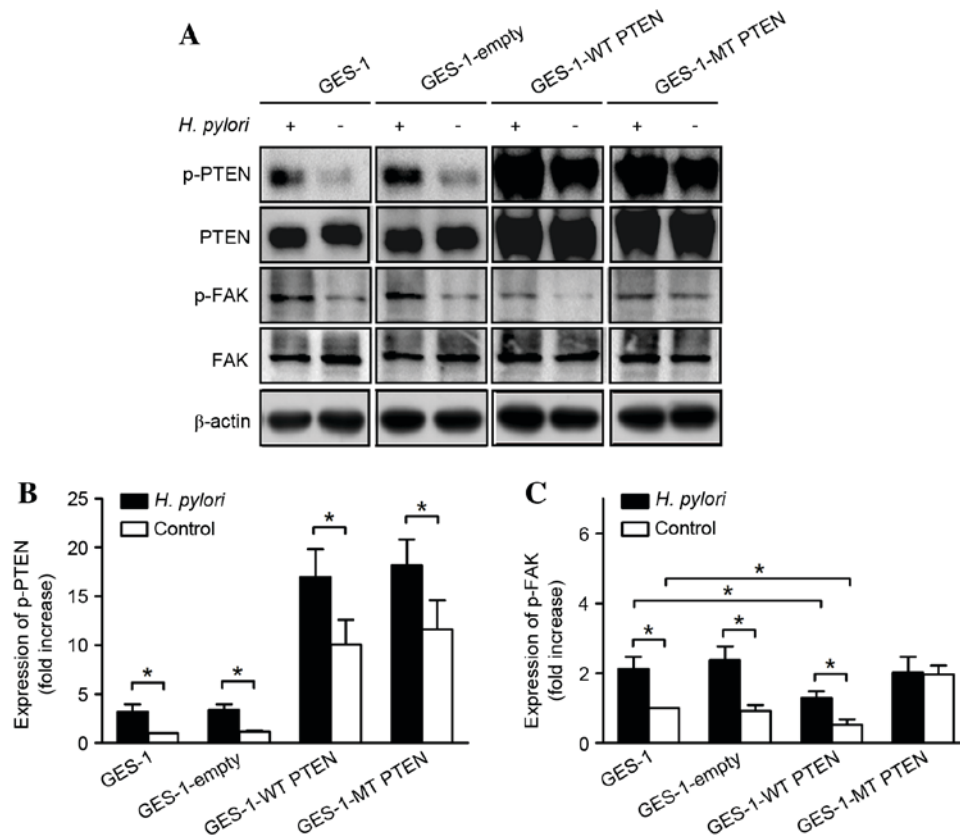
Figure 3. Protein and phosphorylation expression levels of PTEN and FAK following incubation with *H. pylori* in GES-1 gastric epithelial cells overexpressing mt and wt PTEN constructs. (A) Representative western blots from cell lysates of GES-1 cells expressing wt and mt PTEN constructs (GES-1-WT PTEN and GES-1-MT PTEN, respectively) with and without a 1-h incubation with *H. pylori* (multiplicity of infection, 50) are shown. Graphs represent quantitative analysis of (B) p-PTEN and (C) p-FAK expression levels; data are presented as the mean ± standard deviation of triplicated experiments (expressed as the fold of the control). *P<0.05. PTEN, phosphatase and tensin homolog; p-, phosphorylated; FAK, focal adhesion kinase; *H. pylori*, *Helicobacter pylori*; mt, mutant; wt, wild type.

the expression levels of p-PTEN were 1.33±0.62, 3.13±0.34, 3.07±0.58, and 2.85±0.45 at 0.5, 1, 3 and 6 h, respectively (Fig. 2B). Similarly, the expression levels of p-FAK were 1.22±0.22, 2.62±0.30, 2.70±0.35 and 2.65±0.62 relative to 0 h, after 0.5, 1, 3 and 6 h, respectively (Fig. 2C). The increase was determined to be significant after 1-6 h of GES-1 cell incubation with *H. pylori* (P<0.001) without affecting the total FAK protein expression.

Role of PTEN in H. pylori-induced phosphorylation of FAK. Analysis of the western blots revealed that the relative p-PTEN/PTEN expression levels in GES-1 cells transfected with an empty vector were equal to those in the untransfected control GES-1 cells, while the relative expression levels were significantly increased by *H. pylori* infection in all groups (P=0.025) (Fig. 3). Overexpression of wild type PTEN markedly inhibited FAK phosphorylation in the GES-1-wild type PTEN group compared with that in the empty vector and untransfected group; although *H. pylori* increased the levels of p-FAK, this increase was significantly suppressed by overexpression of wild type PTEN, compared with the untransfected group (P=0.023). However, overexpression of an inactive mutant PTEN construct (which mimics phosphorylation at Ser380/Thr382/Thr383) blocked the *H. pylori*-induced increase in p-FAK, without affecting total FAK expression levels. In addition, this overexpression of mutant PTEN increased basal
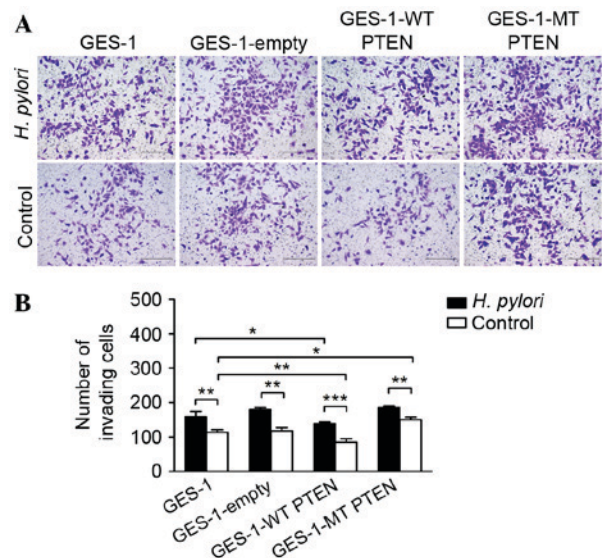

Figure 4. Invasion assay of GES-1 gastric epithelial cells overexpressing mt or wt PTEN constructs following incubation with *H. pylori*. (A) Representative micrographs showing the Transwell invasion of hematoxylin-stained GES-1 cells expressing wt or mt PTEN constructs (GES-1-WT PTEN and GES-1-MT PTEN, respectively), with and without incubation with *H. pylori* (multiplicity of infection, 50; scale bar, 200 μm). (B) Numbers of cells invaded through the membranes are presented as the mean ± standard deviation of triplicated experiments. *P<0.05; **P<0.01; ***P<0.001. PTEN, phosphatase and tensin homolog; p-, phosphorylated; FAK, focal adhesion kinase; *H. pylori*, *Helicobacter pylori*; mt, mutant; wt, wild type.

expression levels of p-FAK, thereby mimicking the effects of *H. pylori* infection.

Role of PTEN in H. pylori-induced cell invasion. Incubation of GES-1 cells with *H. pylori* increased the number of cells that migrated through a Transwell membrane (159.03±15.96 vs. 113.67±7.23; P=0.002; Fig. 4). Similarly, invasion was enhanced by *H. pylori* infection in GES-1 cells transfected with an empty control vector (179.67±6.02 vs. 118.10±9.00; P=0.001), wild type PTEN (138.97±5.00 vs. 85.06±10.00; P=0.001) and mutant PTEN (185.87±4.93 vs. 150.32±7.57; P=0.002). Following *H. pylori* infection, the number of invading cells overexpressing wild type PTEN was decreased compared with the empty vector group (P=0.001). In addition, there was a significant difference among the GES-1-wild type PTEN, mutant PTEN and the untransfected group, as wild type PTEN overexpression decreased the number of invading cells, whereas mutant PTEN expression levels increased the number of invading cells compared with the untransfected group (P=0.004).

Discussion

PTEN is recognized as a frequently inactivated tumor suppressor in a variety of human cancers, and several lines of evidence suggest that abnormal activation of FAK is associated with tumor development (37-39). Continuing from our previous study (28), which described the *H. pylori*-induced phosphorylation of PTEN, the results of the present study demonstrate that gastric cell invasion is promoted by this process via the activation of FAK.

H. pylori infection is one of the most significant identified risk factors for gastric carcinogenesis. Numerous studies use animal models to investigate *H. pylori* infection-associated diseases. The use of various animal species and bacterial strains results in a large variability in the reported results (40). Mongolian gerbils are a unique model due to their low incidence of naturally occurring gastritis and bacterial disease, and have, therefore, commonly been used for studying infection with a standard strain of *H. pylori* (ATCC43504) (41). The results of the current study demonstrate that, concordant with previous results from patients (28), 6-12 months of infection in these gerbils did not significantly alter PTEN expression levels. However, *H. pylori* infection did enhance the expression levels of p-PTEN and p-FAK in gerbils. Although FAK expression levels increased with age, they were not affected by *H. pylori* infection.

The *in vitro* experiments confirmed that *H. pylori*-induced PTEN inactivation promotes FAK phosphorylation, which is consistent with the results of previous studies (34,35). By overexpressing an inactive form of PTEN, where phosphatase activity is eliminated by the mutation of a cysteine to a serine at position 124 (42), the present study demonstrated that the *H. pylori*-induced phosphorylation of FAK is dependent on PTEN activity. In addition, the overexpression of wild type PTEN reduced basal p-FAK levels, indicating enhanced phosphatase activity. These results are concordant with those of a previous study (39) which indicated that overexpression of PTEN inhibits the invasion and metastasis of gastric cancer. However, FAK expression in that study was reported to be downregulated, which was not observed in the current study.

PTEN is known to be crucially involved in the regulation of cell proliferation, growth and survival, and in tumorigenesis (24); however, the role of PTEN in cancer invasion and metastasis is not well understood. The results of the current study demonstrate that *H. pylori* infection significantly increases gastric epithelial cell invasion. Furthermore, cell invasiveness was found to be dependent on PTEN activity, as it was suppressed by wild type, and enhanced by mutant, PTEN overexpression. However, *H. pylori* infection was still able to increase the invasion of cells overexpressing mutant PTEN, suggesting the involvement of an additional signaling pathway, including one that may act directly on the cell cytoskeleton, matrix metalloproteinase-7 or integrins (43-45).

Previous reports have suggested that gastric carcinogenesis is associated with dysregulation of the PTEN signaling pathway (13,14,24,25). The results of the present study demonstrate that *H. pylori* infection enhances this dysregulation by promoting the phosphorylation of PTEN, which subsequently increases FAK activation and gastric epithelial cell invasion. Therefore, we propose that early *H. pylori* infection may influence gastric cancer development by altering signaling pathways important for the morphological changes, adhesion, migration and proliferation of gastric mucosal cells.

Acknowledgements

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