

*H pylori*

## ***H pylori* stimulates proliferation of gastric cancer cells through activating mitogen-activated protein kinase cascade**

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### **Abstract**

**AIM:** To explore the mechanism by which *H pylori* causes activation of gastric epithelial cells.

**METHODS:** A VacA (+) and CagA (+) standard *H pylori* line NCTC 11637 and a human gastric adenocarcinoma derived gastric epithelial cell line BGC-823 were applied in the study. MTT assay and <sup>3</sup>H-TdR incorporation test were used to detect the proliferation of BGC-823 cells and Western blotting was used to detect the activity and existence of related proteins.

**RESULTS:** Incubation with *H pylori* extract increased the proliferation of gastric epithelial cells, reflected by both live cell number and DNA synthesis rate. The activity of extracellular signal-regulated protein kinase (ERK) signal transduction cascade increased within 20 min after incubation with *H pylori* extract and appeared to be a sustained event. MAPK/ERK kinase (MEK) inhibitor PD98059 abolished the action of *H pylori* extract on both ERK activity and cell proliferation. Incubation with *H pylori* extract increased c-Fos expression and SRE-dependent gene expression. *H pylori* extract caused phosphorylation of several proteins including a protein with molecular size of 97.4 kDa and tyrosine kinase inhibitor genistein inhibited the activation of ERK and the proliferation of cells caused by *H pylori* extract.

**CONCLUSION:** Biologically active elements in *H pylori* extract cause proliferation of gastric epithelial cells through activating tyrosine kinase and ERK signal transduction cascade.

### **INTRODUCTION**

*H pylori* is an important pathogen associated with gastritis and peptic ulcers<sup>[1]</sup>. It has also been defined as a carcinogen<sup>[2]</sup>. The mechanisms of pathogenic and carcinogenic effects of *H pylori* infection are under intensive investigation. Research data suggest that *H pylori* might stimulate the proliferation of gastric epithelial cells both *in vitro* and *in vivo*<sup>[3-5]</sup>. This effect of *H pylori* has an important pathogenic significance because increased cell proliferation may elevate the gastric mutation rate and is a predisposing factor for neoplasia. The mechanism, especially the intracellular signal transduction pathway associated with the stimulating effect of *H pylori* on the proliferation of gastric epithelial cells, is under extensive study.

Protein kinases, which regulate the protein phosphorylation, are considered to play the most important role in regulating protein function and cell activity. Mitogen-activated protein kinase (also known as MAP Kinase) is a crucial member of the protein kinase family. So far, four MAP Kinase cascades have been characterized in mammalian cells. Among them, extracellular signal-regulated protein kinase (ERK) cascade is most well characterized. The activation of signal transduction pathways by growth factors, hormones and neurotransmitters is mediated through ERK cascade<sup>[6,7]</sup>. It is the basic signal transduction pathway regulating cell proliferation and differentiation. Recent research data indicate that activation of ERK cascade is involved in *H pylori*-induced proliferation of gastric epithelial cells<sup>[8-10]</sup>. However, more details of *H pylori*-induced activation of ERK cascade are needed to be explored.

### **MATERIALS AND METHODS**

#### **Materials**

Human gastric epithelial cell line BGC-823 (ICLC

HTL98007) was provided by Institute of Tumor Research of Beijing. VacA (+) and CagA (+) international standard *H. pylori* strain NCTC11637 was provided by Institute of Microbiology and Epidemiology of China, Beijing. DMEM culture medium was from Gibco (Grand Island, NY). Brucella broth was from Becton Dickinson (Franklin Lakes, NJ). New born calf serum (NBCS) was from No.2 Factory of Beijing Milk Company (Beijing, China). Antibody against phosphorylated ERK was from Sigma (St. Louis, MO). All other primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). ECL chemoluminescence reagents were from Amersham (Buckinghamshire, England). Tyrosine kinase inhibitors genistein and luciferin were from Sigma (St. Louis, MO). MAPK/ERK inhibitor PD98059 was from Calbiochem (San Diego, CA). Plasmid DNA constructs encoding SRE-luciferase and CRE-luciferase were kind gifts from Dr. Renate Pilz in University of California, San Diego.

### Cell culture

Human gastric cancer cell line BGC-823 was kept in DMEM containing 100 mL/L serum and incubated in 5% CO<sub>2</sub> at 37°C. The medium was changed every second day and the cells were sub-cultured at 80%-90% confluence.

### Preparation of *H. pylori* extract

Healthy *H. pylori* was scraped down from horse serum agar plate and transferred into Brucella broth containing 100 mL/L serum. The bacterial suspension was incubated in micro-oxygen environment (80% N<sub>2</sub>, 15% CO<sub>2</sub>, 5% O<sub>2</sub>) at 37°C for 24 h with continuous shaking. The growth of *H. pylori* was confirmed by Gram staining before harvest. *H. pylori* cells were precipitated by centrifugation at 5000 r/min for 15 min at 4°C and washed with PBS. The bacteria were re-suspended in serum free cell culture medium (DMEM) and sonicated (100 W, 15 s × 6, at 30 s interval). The breakdown of the bacteria was confirmed by microscopy. The suspension was centrifuged at 10000 r/min for 30 min at 4°C. The supernatant (*H. pylori* extract) was adjusted to protein concentration of 1 g/L and kept at -20°C until use.

### Preparation of protein samples

The harvested cells (1 × 10<sup>7</sup>) were washed three times with PBS and re-suspended in 40 μL suspension buffer containing 100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH7.6), 1 mmol/L EDTA (pH8.0), 1 mg/L aprotinin, 100 mg/L PMSF. The same volume of boiled 2 × SDS protein loading buffer containing 100 mmol/L Tris-HCl (pH6.8), 200 mmol/L DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol was added into the cell suspension. The sample was boiled for 10 min to lyse the cells and sonicated for 1 min to break down the DNA. After centrifugation at 12000 r/min for 10 min, the supernatant was kept at -20°C until use. For preparation of membrane protein samples, the cells were harvested, washed with PBS, and lysed with extract buffer containing 20 mmol/L HEPES (pH7.4), 10

mL/L Triton X-100, 5 mmol/L EDTA, 50 mmol/L NaCl, 30 μmol/L β-glycerophosphate, 50 mmol/L sodium fluoride, 50 mg/L aprotinin, 10 mg/L leupeptin. The lysate was centrifuged at 100000 × *g* for 1 h at 4°C and the supernatant was kept at -20°C until use.

### Western blotting

The protein sample was loaded with 30 μg protein per lane on SDS-PAGE gel. After electrophoresis, the proteins on the gel were transferred onto nitrocellulose (NC) membrane as described previously. The NC membrane was blocked with 50 g/L milk in TBS-T for 1 h at room temperature (RT), incubated with first antibody for 2 h and with secondary antibody for 50 min at RT and finally washed three times after each incubation. ECL chemoluminescence reagent was used to show the positive bands on the membrane.

### MTT assay

A total of 1 × 10<sup>4</sup> trypsin-dispersed BGC-823 cells in 0.1 mL culture medium were seeded into each well of the 96-well plates and cultured for 24 h. Then, the cells were incubated with medium alone or with medium plus *H. pylori* extract at different concentrations. After 24 h of further incubation, 10 μL of MTT (6 g/L, Sigma) was added to each well and the incubation was continued for 4 h at 37°C. Finally, the culture medium was removed and 200 μL of dimethylsulphoxide (DMSO) was added to each well. The absorbance was determined with an ELISA reader at 570 nm. The "change percentage" of the absorbency (*A*) value of the cells treated with *H. pylori* extract compared with *A* value of the cells without treatment was calculated as:

$$\text{Change (\%)} = \frac{A \text{ value (} H. pylori \text{ group-control)}}{A \text{ value of control group}} \times 100\%$$

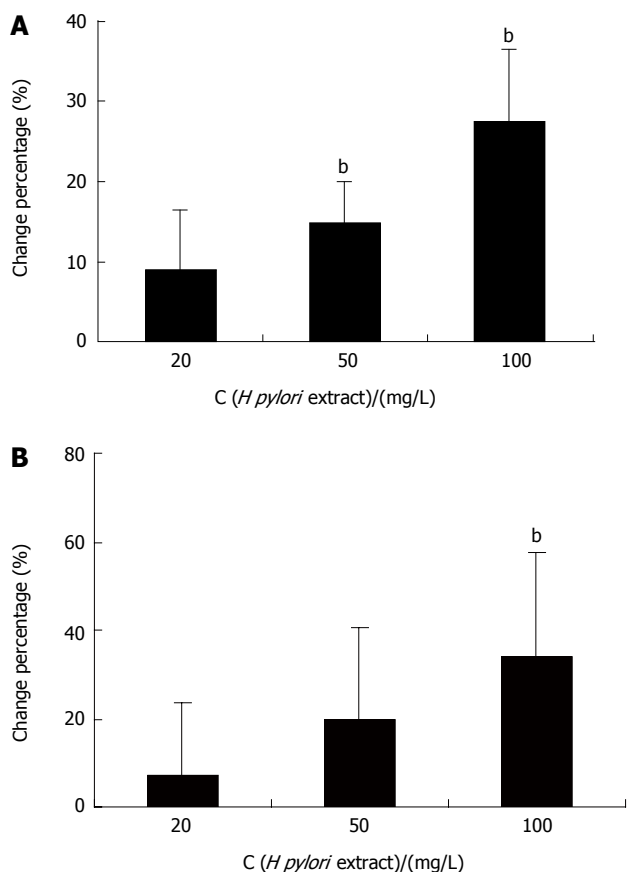
### <sup>3</sup>H-TdR incorporation

The cells were cultured in the same way as described in MTT assay. When the culture medium was changed and *H. pylori* extract was added, 0.2 μCi of <sup>3</sup>H-TdR was also added into each well. After further incubation for 24 h, the medium was discarded and the cells were trypsin-dispersed and collected onto a membrane. The membrane was heated dry at 65°C and put into a plastic vial containing 6 mL of scintillation liquid. The count per minute (CPM) was calculated with a Beckman scintillation counter. The "change percentage" of CPM of the cells treated with *H. pylori* extract compared with CPM of the cells not treated with *H. pylori* extract was calculated as:

$$\text{Change (\%)} = \frac{\text{CPM (} H. pylori \text{ group-control)}}{\text{CPM of control group}} \times 100\%$$

### Reporter gene assay

The cells were transfected with plasmid DNA encoding SRE-luciferase or CRE-luciferase, and β-galactosidase. After incubation with *H. pylori* extract for 8 h, the cells were harvested and the activity of luciferase and β-galactosidase was measured by luminescence-based assays. The luciferase activity was calculated as the fold of increase after normalized by the β-galactosidase activity.



**Figure 1** MTT assay (A) and <sup>3</sup>H-TdR-incorporation test (B) showing *H pylori* extract-stimulated proliferation of BGC-823 cells. <sup>b</sup>*P* < 0.01 vs control.

**Statistical analysis**

The results of MTT assay, <sup>3</sup>H-TdR incorporation test and reporter gene assay data were expressed as means ± SD. The significance of the difference between control group and experimental group was evaluated by Student’s *t*-test.

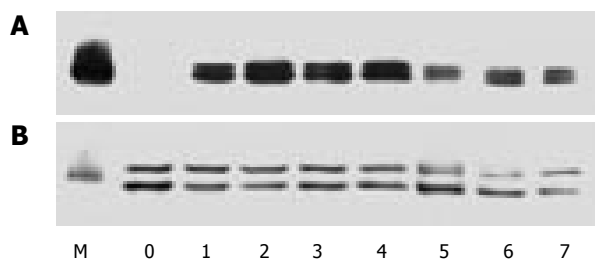
**RESULTS**

***H pylori* extract stimulated proliferation of BGC-823 cells**

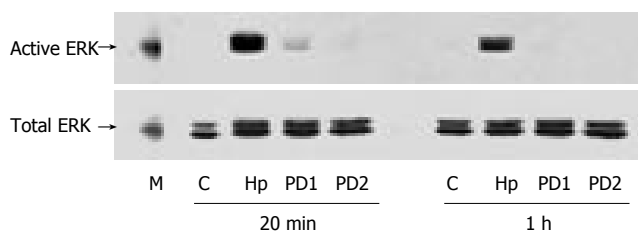
The MTT assay showed that *H pylori* extract could stimulate the proliferation of BGC-823 cells. The *A* value reflecting the number of viable cells, increased significantly in the cells incubated with 50 mg/L and 100 mg/L *H pylori* extract for 24 h (Figure 1A). Similar result was also obtained in <sup>3</sup>H-TdR-incorporation test. The <sup>3</sup>H incorporation increased dose-dependently when the cells were incubated with 20 mg/L, 50 mg/L, and 100 mg/L *H pylori* extract respectively for 24 h (Figure 1B).

***H pylori* extract activated extracellular signal-regulated kinase in BGC-823 cells**

The separated protein samples from cells incubated with 50 mg/L *H pylori* extract for different periods of time were first probed with antibody against phosphorylated ERK to analyze the activity of ERK and re-probed with anti-ERK2 antibody to show the protein content of this kinase. The results showed that in serum-starved cells, the activity level of ERK was very low. During incubation with *H pylori* extract, the activity of ERK increased obviously



**Figure 2** Western blotting showing phosphorylated ERK (A) and total ERK (B) in serum-starved BGC-823 cells incubated with *H pylori* extract. M: Protein molecular marker; 0: Control; 1-7: Incubation with 50 mg/L *H pylori* extract for 20, 40, 60 min and 3, 6, 12, 24 h.



**Figure 3** Western blotting showing PD98059-blocked stimulating effect of *H pylori* extract on ERK activation in BGC-823 cells. M: Molecular marker; C: Control; Hp: 50 mg/L *H pylori* extract; PD1: 25 μmol/L PD98059 + 50 mg/L *H pylori* extract; PD2: 50 μmol/L PD98059 + 50 mg/L *H pylori* extract.

within 20 min and stayed at a high level (about 5 times of control) for more than 8 h (Figure 2).

**PD98059 blocked the stimulating effect of H pylori extract on ERK activity and proliferation of BGC-823 cells**

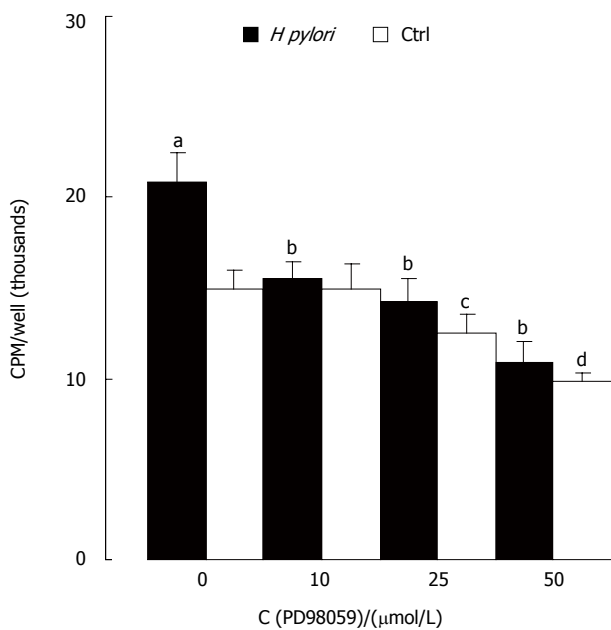
PD98059, an inhibitor of MAPK/ERK kinase (MEK) activating ERK directly, was used to confirm the specificity of ERK activation induced by *H pylori* extract. Western blotting showed that PD98059 abolished the stimulating effect of *H pylori* extract on ERK activity (Figure 3). MTT assay showed that the inhibitor also blocked the stimulating effect of *H pylori* extract on proliferation of the cells (Figure 4).

**Genistein inhibited ERK activation induced by H pylori extract**

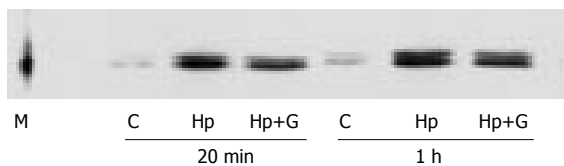
Genistein is an inhibitor of tyrosine kinase. Western blotting with antibody against phosphorylated ERK showed that treating the cells with genistein inhibited the ERK activation induced by *H pylori* extract (Figure 5).

**H pylori extract caused tyrosine phosphorylation of membrane protein**

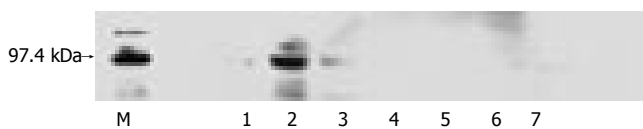
An antibody against pan-tyrosine phosphorylation of proteins was used to detect the tyrosine phosphorylation caused by *H pylori* extract. Western blotting showed that during incubation with *H pylori* extract, several proteins were phosphorylated on tyrosine within 20 min of incubation (Figure 6). A tyrosine-phosphorylated protein with molecular size around 97.4 kDa was especially arrested because it also existed in membrane protein sample extracted from the cells (Data not shown).



**Figure 4** MTT assay showing PD98059-blocked proliferation-stimulating effect of *H. pylori* extract. <sup>a</sup>*P* < 0.05, <sup>c</sup>*P* < 0.05, <sup>d</sup>*P* < 0.01 vs control only; <sup>b</sup>*P* < 0.01 vs Hp only.



**Figure 5** Western blotting showing genistein-prevented ERK activation by *H. pylori* extract. M: Molecular marker; C: Control; Hp: *H. pylori* extract; Hp + G: Genistein and *H. pylori* extract.



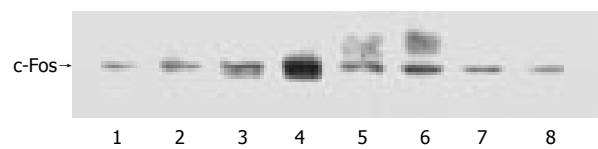
**Figure 6** Western blotting showing *H. pylori* extract-caused tyrosine phosphorylation of cell lysate of BGC-823. M: Molecular marker; 1: control; 2-7: *H. pylori* extract incubated for 20, 40 min and 1, 3, 6, 12 h.

### *H. pylori* extract increased expression of c-fos

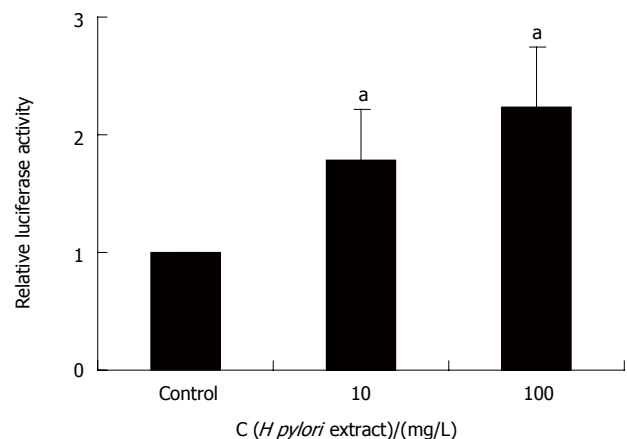
Western blotting showed that when the cells were incubated with 50 mg/L *H. pylori* extract, the expression of c-Fos protein increased obviously. During incubation, the expression of c-Fos started to increase around 40 min after incubation, reached its peak around 1 h and lasted for 6 h (Figure 7).

### *H. pylori* extract stimulated SRE-dependent reporter gene expression

Reporter gene assay showed that *H. pylori* extract specifically stimulated SRE-dependent expression of luciferase while it had no effect on CRE-driven luciferase expression (Figure 8), indicating that *H. pylori* extract could increase c-Fos expression through SRE cis-element of the promoter.



**Figure 7** Western blotting showing *H. pylori* extract-increased expression of c-Fos. 1: Control; 2-8: *H. pylori* extract incubated for 20, 40 min and 1, 3, 6, 12 and 24 h.



**Figure 8** *H. pylori* extract-stimulated SRE-dependent reporter gene expression. <sup>a</sup>*P* < 0.05 vs control.

## DISCUSSION

Gastric *H. pylori* infection is the most common infection in humans. It is responsible for virtually all cases of gastritis and most cases of peptic ulcers<sup>[1]</sup>. Moreover, *H. pylori* is defined as a group 1 carcinogen in humans by International Agency for Research on Cancer, a working party of WHO<sup>[2]</sup>. The conclusion is, however, mainly based on the epidemiological data and clinical investigations. Even though laboratory data on carcinogenesis of *H. pylori* are accumulating during recent years<sup>[11-13]</sup>, more efforts are still needed to elucidate the mechanism of *H. pylori*-induced malignant diseases. Gastric carcinoma develops through a sequence of events from normal mucosa to gastric carcinoma<sup>[14]</sup>. *H. pylori* might be closely associated with this process. Several mechanisms have been proposed by which *H. pylori* infection might lead to predisposition for gastric cancer<sup>[15]</sup>. One explanation is that *H. pylori*-associated chronic inflammation may increase the rate of cell turnover in gastric mucosa. Rapidly replicating DNA is more susceptible to damage. Alongside increased reactive oxygen species and decreased ascorbic acid content during inflammatory reactions, *H. pylori*-induced proliferation may be the major factor for gastric dysplasia and gastric cancer<sup>[16,17]</sup>. In *H. pylori* hosts with gastritis, increased proliferation of gastric epithelial cells has been confirmed<sup>[3,4]</sup>. Laboratory data have also shown that *H. pylori* might directly stimulate proliferation of gastric epithelial cells<sup>[5,10,11]</sup>. The former reveals an increased cell turnover rate in *H. pylori* host and the latter suggests that increased proliferation of gastric epithelial cells might be a direct effect of *H. pylori*.

Probing separated proteins with anti-phosphorylated ERK antibody and re-probing them with anti-ERK2

antibody provide a sensitive way to detect ERK activity<sup>[18]</sup>. With this method, our study showed that during incubation with *H pylori* extract, the activity of ERK increased obviously in serum-starved cells and the activation of ERK was a sustained event. This is significant because sustained activation of ERK appears to be required for many cells to pass the gastrointestinal restriction point to enter S phase, in which cellular DNA is replicated<sup>[19,20]</sup>. MTT assay and <sup>3</sup>H-TdR-incorporation test confirmed the stimulating effect of *H pylori* extract on cell proliferation in our study. Since ERK-mediated cascade is the basic signal transduction pathway regulating cell proliferation, this pathway can mediate proliferation-stimulating effects of *H pylori*.

ERK is in the middle of the signal transduction pathway. Currently, it is believed that ERK may be activated through several pathways<sup>[21]</sup>. One is the receptor tyrosine kinase-Ras-MEK pathway. Growth factor ligand binds to membrane receptor and causes tyrosine-phosphorylation of the receptor. Consequential action of Ras and then Raf, which are components of the cascade, causes the activation of MEK (also known as MAP kinase), a direct upstream regulator of ERK<sup>[22]</sup>. PD98059 is a specific inhibitor of MEK<sup>[23]</sup>. Our results showed that PD98059 could block both ERK activity-stimulating and proliferation-stimulating effects of *H pylori* extract. Ras activity was not investigated in this experiment. However, we used tyrosine kinase inhibitor genistein to explore the possible involvement of tyrosine kinase in *H pylori* extract-induced signaling. The results showed that genistein inhibited *H pylori* extract-induced activation of ERK. Then, we used antibody against pan-tyrosine phosphorylation to detect tyrosine phosphorylation in both whole cell lysate and membrane protein from the cells. The results showed that in samples from the cells treated with *H pylori* extract, there was obvious tyrosine phosphorylation of a membrane protein with molecular size around 97.4 kDa, suggesting that it is very likely that *H pylori* extract activates ERK through receptor tyrosine kinase-Ras-MEK pathway.

As to the down-stream events of ERK activation, it was reported that some ERK activated by MEK can translocate to the nuclei and phosphorylate at least two transcription factors, c-Myc and Elk-1<sup>[24,25]</sup>. The phosphorylated factors may lead to increased production of c-Fos mRNA and finally lead to cell activation<sup>[26]</sup>. Mitsuno *et al*<sup>[27]</sup> reported that co-culturing human gastric cancer cells with Cag-positive *H pylori* strains can activate c-fos gene through SRE. Our results showed that *H pylori* extract stimulated SRE-dependent gene transcription and increased the expression of c-Fos protein, indicating that the signaling process behind activation of ERK by *H pylori* can derive biological elements.

Several methods, including co-culture of live *H pylori* with target cells, use of water extract and sonication extract to stimulate target cells, have been used to study the effect of biologically active components of *H pylori* on the activity of target cells. For example, during co-culture, *H pylori* may secrete active factors or act on the target cells through type IV secretion system<sup>[28]</sup>, water extracting can get the surface component of the bacteria<sup>[29]</sup>, and sonication extracting can efficiently release

active components from the body of bacterial cells<sup>[30,31]</sup>. In this experiment, sonication of *H pylori* extract showed that soluble component from the bacteria activated the MAPK cascade of gastric cells. Further work is needed to identify the molecular features of the component in our laboratory.

In summary, *H pylori* extract has direct proliferation-stimulating effects on gastric epithelial cells and the activation of gastric epithelial cells can be induced by *H pylori* through MAPK-mediated signal transduction pathway, suggesting that biologically active elements in *H pylori* contribute to the tumorigenesis effect of the bacteria.

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