Mol Cell Toxicol (2015) 11:343-348
DOI 10.1007/s13273-015-0034-2

ORIGINAL PAPER

Effects of crude extracted proteins from supernatant of BCF-P and EGF on acid secretion

Yuqing Zhao[1,2], **Tao Guo**[1] **& Jiaming Qian**[1]

Received: 30 October 2014 / Accepted: 15 July 2015

Abstract In order to compare the *in vitro* effects of crude extracts of broth culture filtrate protein (BCF-P) from the supernatant of an *H. pylori* liquid culture and epidermal growth factor (EGF) on acid secretion by isolated rabbit gastric parietal cells. Isolated rabbit gastric parietal cells were incubated with BCF-P or EGF for 1 and 12 h. Morphological changes were assessed by electron microscopy, and ^{14}C-aminopyrine (^{14}C-AP) uptake and H^+-K^+ATP mRNA expression were measured. At 50 μg/mL, BCF-P stimulated acid secretion by parietal cells ($P<0.05$) at 1 h but not at 12 h. At 100 μg/mL, BCF-P significantly inhibited histamine-stimulated acid secretion ($P<0.05$) and decreased H^+-K^+ATP mRNA expression in parietal cells, but it was reversed after EGF exposure. Crude extracted proteins from BCF-P damaged the inner cell membrane structure of parietal cells, reduced H^+-K^+ATP gene expression, and inhibited acid secretion, activities that differed markedly from those of EGF.

Keywords Gastric parietal cells, *Helicobacter pylori*, Vacuolating cytotoxin, Acid secretion, H^+-K^+ATP enzyme

The supernatant of *Helicobacter pylori* growth medium contains a toxin that induces vacuolar degeneration in eukaryotic cell lines *in vitro*[1]. This toxin, called vacuolating cytotoxin (VacA)[2], was demonstrated to be the primary pathogenic virulence factor of *H. pylori* that can inhibit acid secretion by gastric parietal cells. Although the factor is excreted by *H. pylori* into culture supernatant, it is difficult to purify. Thus, there have been few studies of the pathogenic mechanism of VacA.

Gastric mucosal cells secrete gastric acid, relying on the apical membrane proton pump H^+-K^+ATP enzyme to actively transport H^+ against a concentration gradient. Parietal cell preparation is relatively difficult, limiting studies of acid secretion by these cells. We have successful isolated parietal cells and grown them in primary culture through elutriation and continuous density gradient centrifugation[3]. The purity of these cell preparations is approximately 80-90%, making it possible to study the effects of various exogenous stimulating factors on acid secretion. We therefore investigated the effects of *H. pylori* VacA on acid secretion by gastric parietal cells *in vitro*.

Identification of vacuolating activity in BCF-P

SDS-PAGE revealed BCF-P bands at a molecular weight of 95 kDa corresponding to VacA protein[2] (Figure 1). Different batches of cells yielded similar bands. Serial dilutions of BCF-P (from 1 : 2 to 1 : 256) revealed that VacA activity was maximal at 1 : 8, corresponding to a protein concentration of 100 μg/mL. The HeLa cell cytoplasmic vacuoles were of different, uneven sizes, and they could fuse to form large bubbles (Figure 2).

Isolation and culture of rabbit parietal cells, and identification of their activities

Parietal cells were isolated from the rabbit gastric body and mucosa bottom by elutriation and separation, followed by density gradient centrifugation with 50% Percoll. The parietal cell purity was 80.5%. Parietal cells started to adhere to the walls of the flasks after culture for 2 h; at 6-8 h after inoculation, the adherent

[1]Department of Gastroenterology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Beijing 100730, China
[2]Deportment of VI P, General Hospital of Beijing Command, People's Liberation Army, Beijing 100700, China
Correspondence and requests for materials should be addressed to J. Qian (✉jiaming923@sina.com)

parietal cells formed single circles of uniform distribution. Trypan blue exclusion revealed a survival rate of 90-95%, and their morphology was assessed by fluorescence microscopy after 5 min of staining with acridine orange. Incubation of isolated parietal cells with 1.0×10^{-6}, 1.0×10^{-5}, and 1.0×10^{-4} mol/L histamine for 30 min yielded relative ^{14}C-AP uptakes of (3.185 ± 0.286), (5.073 ± 0.311), and (9.123 ± 1.315), respectively, all significantly higher than that of control cells (1.000; each $P < 0.05$). Moreover, uptake was dependent on the histamine concentration, indicative of good acid secretion activity.

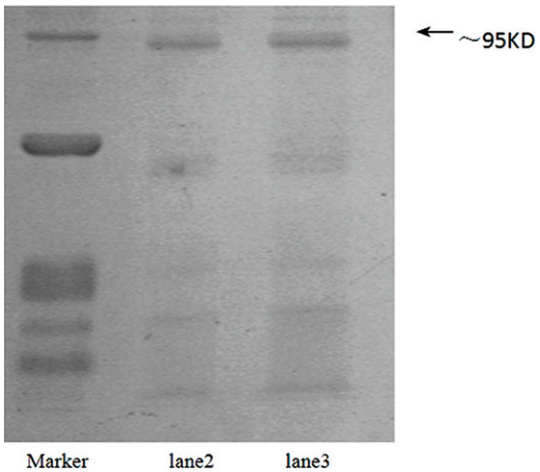


Figure 1. SDS-PAGE of crude extracted proteins from *H. pylori* broth supernatant. Lane 1, marker proteins; lanes 2 and 3, BCF-P samples.

Transmission electron microscopy

Electron microscopy of normal parietal cells disclosed that their nuclei were circular or elliptical and located in the center of each cell. The cytoplasm contained tortuous branch endocrine small tubes, and the cell walls and top surface of the plasma membrane were connected by microvilli. The parietal cells had a large number of organelles but few mitochondria (Figure 3a). After treatment with 100 μg/mL BCF-P for 1 h, the tubular structures of these parietal cells were damaged, the nucleus was pushed to one side, and a large of used bubble (Figure 3b) appeared in the cytoplasm. These activities were not observed in cells incubated with EGF (not shown).

Effects of BCF-P and EGF on histamine-stimulated acid secretion by parietal cells

Incubation of cells with 100 μg/mL BCF-P for 1 h or

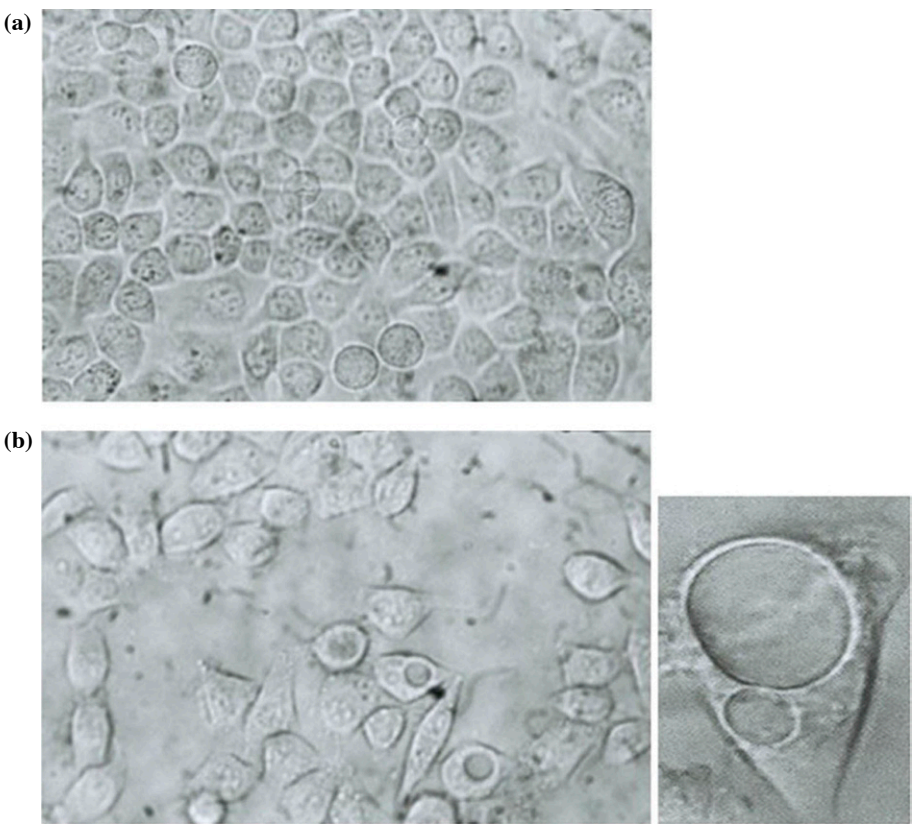
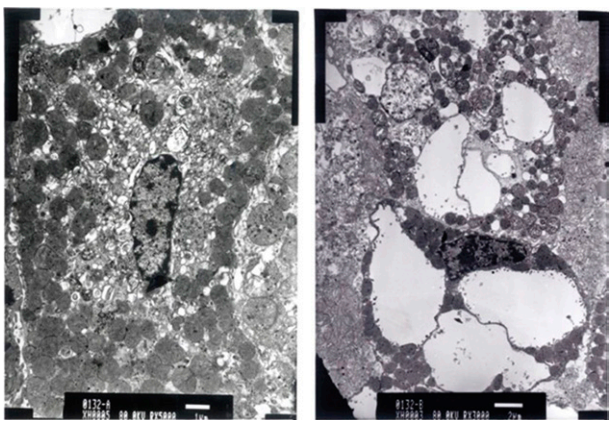


Figure 2. Inverted microscopy of HeLa cells. (a) Normal HeLa cells. (b) Inverted microscopy of HeLa cells with vacuolar degeneration (10 × 40) after incubation for 6 h with 100 μg/mL BCF-P.

12 h significantly inhibited histamine-induced acid secretion, an inhibition that increased over time and differed significantly from that in cells incubated with histamine alone ($P=0.000$). By contrast, incubation with 50 μg/mL BCF-P for 1 h significantly enhanced histamine-induced acid secretion ($P=0.000$), but after 12 h, there was no difference from control cells incubated with histamine alone ($P=0.582$). Incubation with 50 ng/mL EGF for 1 h inhibited acid secretion, whereas incubation for 12 h stimulated gastric acid secretion ($P=0.000$) (Table 1).

Effects of BCF-P and EGF on H^+-K^+ATPase mRNA expression

After treatment for 1 or 12 h, BCF-P (100 ng/mL) significantly inhibited the expression of H^+-K^+ATPase α subunit mRNA ($P=0.000$), as did BCF-P treatment (50 μg/mL) for 12 h ($P=0.001$) but not for 1 h ($P=0.509$). The effects of BCF-P on H^+-K^+ATPase α subunit mRNA expression in parietal cells were both time- and concentration-dependent. Conversely, EGF (50 ng/mL) upregulated H^+-K^+ATPase α subunit mRNA after treatment for 1 or 12 h ($P<0.05$ each; Figure 4).



(a) (b)

Figure 3. Electron microscopy of rabbit parietal cells cultured *in vitro*. (a) Normal cells (×5000 magnification); (b) after treatment with 100 μg/mL BCF-P for 1 h (×3000 magnification).

Discussion

H. pylori is a gram-negative bacterium that colonizes approximately half of the world's population, and it is consistently associated with gastric mucosal inflammation, ulcers, and gastric cancer[8,9]. One of the major virulence factors of *H. pylori* the vacuolating toxin VacA[10,11]. VacA protein has been associated with the *in vivo* long-term colonization of *H. pylori*, and it can cause direct damage to gastric epithelial cells, eventually resulting in peptic ulcers and tumors. The *H. pylori* VacA toxin has been found to impair gastric parietal cell physiology by disrupting the apical membrane-cytoskeletal linker, preventing the recruitment and fusion of H^+-K^+ATPase-containing tubulo vesicles, and causing hypochlorhydria[12]. The *H. pylori* cag PAI gene products CagE, CagM, CagL, and possibly CagA were found to be mechanistically involved in the re-expression of the H^+-K^+ATPase α subunit. Further, acute *H. pylori* infection of the human gastric mucosa down-regulates parietal cell H^+-K^+ATPase expression, significantly inhibiting acid secretion[13]. We tested the hypothesis that *H. pylori* VacA could act directly on gastric parietal cells. We also found that the effects of the VacA toxin on acid secretion by parietal cells differed from those of EGF.

To obtain the *H. pylori*-secreted proteins, it was necessary to culture *bacteria* in a liquid medium. We found that optimal conditions included 0.5% β-CD as the supporting agent, an inoculation density of 10^8 cfu/mL, and culture in Brucella broth medium for 72 h. Under

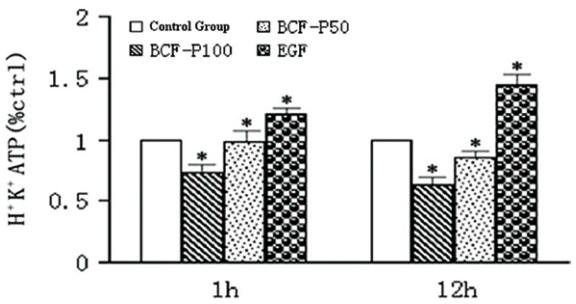


Figure 4. Effects of BCF-P and EGF on H^+-K^+ATPase α subunit mRNA expression in rabbit parietal cells.

Table 1. Effects of BCF-P and EGF at different concentration on histamine-stimulated acid secretion by parietal cells.

^{14}C-AP Accumulation	BCF-P (100 μg/mL), % control	BCF-P (50 μg/mL), % control	EGF (50 μg/mL) % control
1 h	0.756 ± 0.081*	1.368 ± 0.076*	0.604 ± 0.039*
12 h	0.423 ± 0.063*	0.977 ± 0.088	1.898 ± 0.167*

Cells were incubated with 1×10^{-4} M histamine and with BCF-P or EGF as shown. All results are presented as the mean ± SD, and they were calculated as follows: ^{14}C-AP accumulation (% control) = (CPM in an experimental group − CPM in DNP group)/(CPM of control group − CPM in DNP group). *$P<0.05$ vs. control by Student's t-test (n = 5).

these conditions, the largest quantities of intact *bacteria* could be harvested. Semi-saturated ammonium sulfate precipitation did not destroy the natural configuration of proteins. We found that a protein band of approximately 95 kDa in size that corresponded to VacA, with neutral red uptake illustrating that BCF-P could induce vacuolar degeneration in HeLa cells and directly damage the tubular structures of parietal cells.

Parietal cells are highly differentiated epithelial cells with unique membrane structures and plentiful mitochondria that have secreting functions. H^+-K^+ATPase, the proton pump of parietal cells, is composed of two subunits, an α subunit with catalytic activity and a β subunit that imparts structural stability. Reductions in H^+-K^+ATPase expression may reduce acid secretion by these cells. Using RT-PCR, we compared the effects of BCF-P and EGF on the expression of H^+-K^+ATPase α subunit mRNA by isolated parietal cells. Endogenous EGF does not suppress acid secretion *in vivo*. *In vitro*, however, EGF inhibits acid secretion by isolated parietal cells in the short term, followed by stimulation in the long term. Short-term inhibition may act through the PKC pathway, whereas longer-term stimulation may increase H^+-K^+ATPase gene expression through the ERKs and/or Akt pathways[14-17].

H. pylori VacA may interfere with EGF-activated signal transduction pathways[18,19]. Activation of EGFR then initiates a cascade of intracellular signaling pathways, including activation of the ERK/MAP kinase cascade, ultimately leading to the activation of transcription factor activator-protein 1, which plays a crucial role in cell proliferation and transformation. EGFR, as the *H. pylori* VacA receptor, may mediate endocytosis of the toxin, allowing it to enter cells[20]. *In vitro* experiments have also revealed that VacA acts on the gastric adenocarcinoma cell line KATO III by inhibiting EGF-induced downstream physiological effects[21].

We found that EGF inhibited, and then stimulated, acid secretion by parietal cells, with similar effects on the expression of H^+-K^+ATPase α subunit mRNA. By contrast, the effects of BCF-P were in a manner of concentration and time dependent. The lower concentration of BCF-P mildly stimulated acid secretion during the acute phase, but it had no effect at later times. This same concentration had no effect on H^+-K^+ATPase α subunit mRNA expression during the acute phase, but it down-regulated its expression at later times. Conversely, a higher concentration of BCF-P significantly inhibited acid secretion and down-regulated H^+-K^+ATPase α subunit mRNA expression during both the acute and chronic phases. Electron microscopy disclosed that BCF-P caused vacuolar degeneration and seriously damaged the inner membrane structure of parietal cells. Thus, BCF-P-mediated inhibition of gastric acid secretion may be due to direct damage to the inner membrane structure of parietal cells and reduced H^+-K^+ATPase gene expression, a mechanism of action completely different from that of EGF.

At a lower concentration, the acute action of BCP-F is relatively weak and insufficient to cause such lesions in parietal cells. Its ability to stimulate gastric acid secretion during the acute phase may be related to the characteristics of VacA protein. The VacA polypeptide chain is easily cleaved at a protease-sensitive loop to form a dimer of two subunits (p34 and p58). Moreover, VacA can assemble in hexameric rosettes, in which p58 forms spike-like protrusions on the outside and p34 forms the internal core; under appropriate conditions, VacA can also form heptamers, dodecamers, or tetradecamers. In membranes, these complexes can form anion channels with low conductivity and apparently with a preference for chloride ions[22]. These channels can be blocked by the typical Cl^- ion channel blocker DIDS[23]. These same Cl^- channels were observed on HeLa cell membranes, different from the original endogenous channels in the cells[24]. Further experiments are required to confirm whether lower concentrations of BCF-P can use VacA-formed Cl^- ion channels to transiently stimulate acid secretion by parietal cells and whether the time- and concentration-dependent effects of BCF-P (VacA) act through different pathways.

In summary, the crude extracted protein from the supernatant of *H. pylori* liquid cultures contains VacA protein, which exerts in vitro vacuolating toxin activity in HeLa cells and directly inhibits acid secretion by parietal cells by damaging the inner cell membrane structure and down-regulating H^+-K^+ATPase expression. The inhibition induced by VacA is completely different from that of EGF.

Materials & Methods

H. pylori liquid culture and BCF-P extraction

The *H. pylori* virulence strain *NCTC 11637*, which is positive for cytotoxin-associated protein (Cag A) and VacA, was provided by the Institute of Communicable Disease Control and Prevention at the China Center of Disease Control and Prevention. The *NCTC 11637* strain was inoculated onto Columbia agar medium by the crossing line method and cultured for 2-3 days under a microaerophilic environment, consisting of 5% O_2, 85% N_2, and 10% CO_2. The bacteria were subsequently inoculated at a density of 10^8 cfu/mL into Brucella broth medium containing 0.5% of the support agent β-CD and cultured with shaking at 120 rpm for

72 h in the microaerophilic environment at 37°C.

HeLa cell culture and broth culture filtrate protein (BCF-P) vacuolating activity[4]

The liquid supernatant of the *H. pylori* culture was collected; BCF-P was extracted by ammonium sulfate half-saturated precipitation, concentrated, and dried under vacuum. SDS-PAGE was performed to detect a protein with the molecular weight of VacA, and the Lowry method was used for protein quantification. BCF-P vacuolar activity was assayed by the HeLa cell neutral red uptake test, which was conducted by Interagency Coordinating Committeeonthe Validation of Alternative Methods (ICCVAM).

Separation and identification of isolated rabbit gastric parietal cells[5]

Male New Zealand rabbits were provided by the Animal Laboratory at Beijing Union Medical College Hospital, Chinese Academy of Medical Sciences. Rabbit gastric mucosae were digested with collagenase I (300 mg/L), and parietal cells were obtained from single-cell suspensions by separation with a Beckman JE-6B elutriation machine. Survival was assessed by Trypan blue staining, and cell morphology was evaluated using fluorescence microscopy following acridine orange fluorescence staining. Parietal cells were inoculated into a poly-L-lysine-pre-coated culture flask to allow cells to adhere.

Transmission electron microscopic observations of changes in parietal cell morphology

The isolated parietal cells were cultured for 6 h, and the medium was replaced with new parietal cell complete medium containing BCF-P (100 μg/mL) or epidermal growth factor (EGF, 50 ng/mL). After another hour, the cells were fixed by adding 1 mL of freshly prepared 2.5% glutaraldehyde for more than 1 h at 4°C. The cells were dehydrated in an acetone gradient at room temperature and embedded in embedding agent; after ultrathin sectioning, the cells were stained with uranyl acetate and lead citrate, and viewed using electron microscopy.

^{14}C-Aminopyrine (^{14}C-AP) uptake[6]

BCF-P (50 or 100 μg/mL) or EGF (50 ng/mL) was added to parietal cells cultured *in vitro* for 6 h; as a control, 2,4-dinitrophenol (DNP) was added to correct for the non-specific adsorption of ^{14}C-AP for 1 or 12 h at 37°C in an atmosphere containing 5% CO_2 and saturated humidity. Acid secretion was determined using the ^{14}C-AP uptake method; results were expressed as the relative uptake of ^{14}C-AP.

Expression of H^+-K^+ATPase mRNA

BCF (50 or 100 μg/mL or EGF (50 ng/mL) was added to parietal cells cultured for 6 h at 37°C in 5% CO_2 and saturated humidity for 1 or 12 h. Total RNA was extracted using the TRIzol method, and RT-PCR was used to measure H^+-K^+ATPase mRNA expression with ubiquitin carboxyl terminal precursor (UBCP) mRNA as an internal reference. The H^+-K^+ATPase α chain primers were 5′-ACTCTGGGCTCCACGTCG-3′ (forward) and 5′-AGGATGGAGCTGCAGCGC-3′ (reverse), yielding a 470-bp PCR product; the UBCP[7] primers were 5′-AGAAGAAGTCTTACACCACTC-3′ (forward) and 5′-GTAAGTCAGACAACATTTGCC-3′ (reverse), yielding a 203-bp PCR product. The amplification conditions consisted of initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and denaturation at 72°C for 1 min, and a final extension at 72°C for 10 min. In all samples, the relative abundance of H^+-K^+ ATPase mRNA was calculated by normalization relative to the expression of UBCP mRNA.

Statistical analysis

All statistical analyses were performed using SPSS 13.0 statistical software. Each experiment was repeated at least three times, with the results expressed as the mean ± standard deviation. Differences between two groups were compared using Student's t-tests, and differences among three or more groups were compared using single-factor variance analysis. $P < 0.05$ was defined as statistically significant.

Author contributions

ZYQ carried out the design and coordinated the study, participated in most of the experiments and prepared the manuscript. GT provide assistance in the design of the study, coordinated and carried out all the experiments and participated in manuscript preparation. QJM provided assistance for all experiments. All authors have read and approved the content of the manuscript.

Ethical aspects of animal experiments All animal procedures were performed in accordance with the protocols 0520 approved by the Committee for Supervision of Animal Experiments in the Beijing Military General Hospital Ethics Committee.

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Leunk, R. D., Johnson, P. T., David, B. C., Kraft, W. G. & Morgan, D. R. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *J Med Microbiol* **26**:93-99 (1988).
2. Cover, T. L. & Blaser, M. J. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J Biol Chem* **267**:10570-10575 (1992).
3. Li, X. B., Qian, J. M. & Chen, Y. F. Separation and application of parietal cells. *J Gastroenterol Hepatol* **5**: 254-26 (1996) (in Chinese).
4. Papini, E. *et al*. Cellular vacuoles induced by *Helicobacter pylori* originate from late endosomal compartments. *Proc Natl Acad Sci USA* **91**:9720-9724 (1994).
5. Li, X. B., Qian, J. M., Chen, Y. J. & Chen, Y. F. The mechanism of somatostatin-induced acid secretion inhibition in isolated parietal cells. *Chin J Intern Med* **40**: 236-238 (2001) (in Chinese).
6. Soll, A. H. Secretagogue stimulation of 14Caminopyrine accumulation by isolated canine parietal cells. *Am J Physiol* **238**: G366-G375 (1980).
7. Campbell, V. W. & Yamada, T. Acid secretagogue-induced stimulation of gastric parietal cells gene expression. *J Biol Chem* **264**:11381-11386 (1989).
8. Cover, T. L. & Blaser, M. J. *Helicobacter pylori* in health and disease. *Gastroenterology* **136**:1863-1873 (2009).
9. Suerbaum, S. & Michetti, P. *Helicobacter pylori* infection. *N Engl J Med* **347**:1175-1186 (2002).
10. de Bernard, M., Cappon, A., Del Giudice, G., Rappuoli, R. & Montecucco, C. The multiple cellular activities of the VacA cytotoxin of *Helicobacter pylori*. *Int J Med Microbio* **293**:589-597 (2004).
11. Jones, K. R., Whitmire, J. M. & Merrell, D. S. A Tale of Two Toxins: *Helicobacter pylori* CagA and VacA Modulate Host Pathways that Impact Disease. *Front Microbiol* **115** (2010).
12. Wang, F. *et al*. *Helicobacter pylori* VacA disrupts apical membrane-cytoskeletal interactions in gastric parietal cells. *J Biol Chem* **283**:26714-26725 (2008).
13. Saha, A., Hammond, C. E., Beeson, C., Peek, R. M. Jr, & Smolka, A. J. *Helicobacter pylori* represses proton pump expression and inhibits acid secretion in human gastric mucosa. *Gut* **59**:874-881 (2010).
14. Nakamura, K., Zhou, C. J., Parente, J. & Chew, C. S. Parietal cell MAP kinases: multiple activation pathways. *Am J Physiol* **271**:G640-G649 (1996).
15. Chew, C. S., Nakamura, K. & Petropoulos, A. C. Multiple actions of epidermal growth factor and TGF-alpha on rabbit gastric parietal cell function. *Am J Physiol* **267**:G818-G826 (1994).
16. Joshi, V., Ray, G. S. & Goldenring, J. R. Inhibition of parietal cell acid secretion is mediated by the classical epidermal growth factor receptor. *Dig Dis Sci* **42**:1194-1198 (1997).
17. Finke, U., Rutten, M., Murphy, R. A. & Silen, W. Effects of epidermal growth factor on acid secretion from guinea pig gastric mucosa: in vitro analysis. *Gastroenterology* **88**:1175-1182 (1985).
18. Pai, R. *et al*. *Helicobacter pylori* culture supernatant interferes with epidermal growth factor-activated signal transduction in human gastric KATO III cells. *Am J Pathol* **152**:1617-1624 (1998).
19. Caputo, R. *et al*. *Helicobacter pylori* VacA toxin upregulates vascular endothelial growth factor expression in MKN 28 gastric cells through an epidermal growth factor receptor-, cyclooxygenase-2-dependent mechanism. *Clin Cancer Res* **9**:2015-2021 (2003).
20. Seto, K., Hayashi-Kuwabara, Y., Yoneta, T., Suda, H. & Tamaki, H. Vacuolation induced by cytotoxin from *Helicobacter pylori* is mediated by the EGF receptor in HeLa cells. *FEBS Lett* **431**:347-350 (1998).
21. Pai, R. *et al*. *Helicobacter pylori* culture supernatant interferes with epidermal growth factor-activated signal transduction in human gastric KATO III cells. *Am J Pathol* **152**:1617-1624 (1998).
22. Antoine, G. & Joachim, R. Targeting of *Helicobacter pylori* VacA to mitochondria. *Gut Microbes* **1**:392-395 (2010).
23. Iwamoto, H., Czajkowsky, D. M., Cover, T. L., Szabo, G., Shao, Z. VacA from *Helicobacter pylori*: a hexameric chloride channel. *FEBS Lett* **450**:101-104 (1999).
24. Szabò, I. *et al*. Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of *Helicobacter pylori* is required for its biological activity. *EMBO J* **18**:5517-5527 (1999).