Human gastric fibroblasts ameliorate A20-dependent cell survival in co-cultured gastric epithelial cells infected by *Helicobacter pylori*

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Abstract

Crosstalk between the gastric epithelium, which is closely in contact with stromal fibroblasts in the gastric mucosa has a pivotal impact in proliferation, differentiation and transformation of the gastric epithelium. The human pathogen *Helicobacter pylori* colonises the gastric epithelium and represents a risk factor for gastric pathophysiology. Infection of *H. pylori* induces the activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is involved in the pro-inflammatory response but also in cell survival. We found in co-culture of human gastric fibroblasts (HGF) that apoptotic cell death is suppressed in polarised human gastric cancer cell line NCI-N87 or gastric mucosoids during *H. pylori* infection. Interestingly, suppression of apoptotic cell death in NCI-N87 cells involved an enhanced A20 expression, which was regulated by NF-κB activity in response to *H. pylori* infection. Moreover, A20 acts as an important negative regulator of caspase-8 activity, which was suppressed in NCI-N87 cells during co-culture with gastric fibroblasts. Our results provide evidence for a NF-κB-dependent regulation of apoptotic cell death in cellular crosstalk and underscore the protective role of gastric fibroblasts on gastric epithelial cell death during *H. pylori* infection.

Keywords; caspase-8, gastric cancer, gastric mucosoids, gastric organoids, NF-κB

1. Introduction

The epithelium of the gastric mucosa contains different cell types with distinct functions, including mucus-producing cells, acid-secreting parietal cells and pepsinogen-secreting chief cells [1]. The lamina propria, a loose connective tissue under the gastric epithelium, contains various surrounding stromal cells, including fibroblasts, vascular endothelial, smooth muscle cells, and immune cells [2]. Remarkably, emerging evidence suggests critical functions of fibroblasts that go beyond their fundamental role as structural scaffolds, including the control of cell survival, differentiation, and migration [3]. However, the effect of fibroblasts on epithelial cell survival remains a controversy. It has been suggested that different types of fibroblasts may release diverse factors that influence proliferation, apoptosis, and drug response of epithelial cells [4,5]. Thus, fibroblasts may function as either positive or negative regulators of epithelial cell growth depending on the type of fibroblast. Interestingly, the co-culture of murine glandular stomach cells and gastric mesenchymal fibroblasts revealed that the gastric fibroblasts contribute to the long-term maintenance of stem cell activity and increase the differentiation and proliferation of the gastric epithelium [6]. This finding suggests the

importance of gastric fibroblasts for maintaining the functional integrity of the gastric epithelium in the gastric mucosa.

The gastric epithelium represents a barrier protecting the stomach from external agents, including pathogens. However, a colonising of *H. pylori* bacterium exists in nearly half of the world's population [7]. *H. pylori* is also a risk factor for stomach diseases such as peptic ulcers, chronic gastritis, and gastric cancer [8]. Furthermore, infection with *H. pylori* is associated with significant gastric epithelial cell damage, including apoptotic cell death pathway [9-17]. Herein, the *H. pylori*-induced NF-κB pathways and their target genes encoding cell survival factors (cIAP1, cIAP, A20) play a crucial role [18].

Although the presence of fibroblasts underneath the human stomach epithelium is widely known, its crosstalk with the overlaying epithelium has still not been well characterised. In particular, it is unclear if the fibroblasts affect survival of gastric epithelial cells during *H. pylori* infection. Here, we examined the effect of gastric fibroblasts on the survival of polarised gastric epithelial cancer cells and primary gastric mucosoids. We identified a protective role of the fibroblasts on the epithelial cell death during *H. pylori* infection, and suggest that NF-κB-regulated A20 contributes this process.

2. Materials and methods

2.1. Cell culture and *H. pylori* infection

The human NCI-N87 gastric carcinoma cell line (CRL-5822, ATCC) was cultured as polarised cell monolayer. 2 × 10^5 cells were seeded on a porous membrane (pore size 1 µM) of 12-well ThinCert™ inserts (Greiner Bio-One). The cells were further cultivated for 4 days in RPMI 1640 medium (Gibco®/ Life Technologies) supplemented with 10% fetal calf serum (FCS; Biochrom) at 37 °C in a humidified 5% CO_2 atmosphere to obtain a confluent polarised cell monolayer. Human gastric fibroblasts (HGF; #2830, ScienceCell™ Research Laboratory) were seeded at 2 × 10^5 cells per well in 12-well plate and cultured in RPMI 1640 medium supplemented with 10% FCS at 37 °C in a humidified 5% CO_2 atmosphere for 4 days. The culture medium was replaced with fresh RPMI 1640 medium supplemented with 10% FCS overnight before starting the co-culture experiment and infection.

For gastric mucosoid cultures, 4 × 10^5 cells derived from gastric organoids [19] were seeded onto a collagen-coated (15 µg/cm^2, A10644-01, Gibco®) membrane of a 12-well ThinCert™ insert. The cells were cultivated in a mucosoid culture medium (advance DMEM/F12 ++ (12634-010, Thermo Fisher Scientific) and 25% (v/v) R-Spondin conditioned medium supplemented with 25ng/ml Wnt Surrogate-Fc Fusion Protein (N001, U-Protein Express B.V.), 2% (v/v) B-27™ Supplement (50X) (17504-044, Thermo Fisher Scientific), 10 mM

Nicotinamide (N0636-100G, Sigma Aldrich), 1% (v/v) Penicillin-Streptomycin (100X) (15140-122, Thermo Fisher Scientific), 1% (v/v) N-2 Supplement (100X) (17502-048, Thermo Fisher Scientific), 20 ng/ml Human EGF (PHG0311, Thermo Fisher Scientific), 1 uM TGF-β RI Kinase Inhibitor IV (Alk-I) (616454, Calbiochem), 150 ng/ml Human FGF-10 (100-26, PeproTech), 150 ng/ml Human Noggin (120-10C, PeproTech), 10 nM Human [Leu^{15}]-Gastrin I (G9145, Sigma Aldrich) and 7.5 µM ROCK inhibitor (Y-27632) (Y0503, Sigma Aldrich)) at 37 °C in a humidified 5% CO_2 atmosphere.

The medium overlaying the cells was removed on day 4 after seeding to generate an air-liquid interface (ALI) culture. The medium at the basolateral side was then replaced with a mucosoid culture medium supplemented with 1.5 µM ROCK inhibitor twice a week. Under ALI culture conditions, the mucosoids produce and accumulate mucous on the apical side, which was removed twice a week. The gastric mucosoids were cultivated for 18 days to form a monolayer with complete barrier integrity [19]. The mucous was removed and the mucosoids were washed twice with PBS containing calcium and magnesium before starting the co-culture experiment and infection.

For the co-culture experiment, the ThinCert™ inserts containing confluent polarised NCI-N87 cells or gastric mucosoids were hung in the 12-well plate on top of the HGF cells.

For collecting the conditioned media, the NCI-N87 and HGF cells were seeded on a membrane of ThinCert™ inserts and in a 12-well plate, respectively. The cells were cultured in three different conditions as follows: (1) HGF mono-culture, (2) HGF in co-culture with polarised NCI-N87 cells and (3) HGF in co-culture with *H. pylori*-infected polarised NCI-N87 cells. After 18 h, the culture medium of HGF in the basolateral compartment (conditioned medium) was collected, centrifuged at 600g for 10 minutes, and filtered through 0.2-micron filters.

H. pylori strain P1 [20] was grown on GC agar plates supplemented with 10% horse serum (Gibco®/Life Technologies), 5 µg/ml trimethoprim (Sigma-Aldrich), 1 µg/ml nystatin (Sigma-Aldrich), 10 µg/ml vancomycin (Sigma-Aldrich) under microaerophilic conditions at 37 °C for 48 h prior infection. Cells were infected with *H. pylori* at MOI 100.

2.2. Assessment of cell monolayer integrity

2.2.1. TEER measurement

Measurement of transepithelial electrical resistance (TEER) was performed using a Millicell electrical resistance system (Millipore) according to the manufacturer's instruction. TEER values were calculated as kOhm (kΩ) × cm^2. The cell monolayers reaching TEER values above 0.5 kΩ × cm^2 were considered to have an appropriate barrier function and used for further study.

2.2.2. FITC dextran measurement

The paracellular permeability of the cell monolayer was evaluated by measuring the diffusion of fluorescein isothiocyanate (FITC)-labelled dextran (molecular mass 4 kDa, Sigma) from the apical to the basolateral medium compartment. The apical culture medium was replaced by a medium containing FITC-dextran (1mg/ml), whereas the basolateral medium was changed to a fresh medium without FITC-dextran. After incubation at 37 °C for 4 h, aliquots were collected from apical and basolateral compartments. The fluorescence intensity of the collected media was measured using a multi-mode plate reader (SpectraMax M5, Molecular Devices) with an excitation wavelength of 492 nm and an emission wavelength of 520 nm. The cell monolayer's permeability was presented as a percentage of FITC-dextran transported across the gastric monolayer compared to control (blank membrane).

TEER and FITC-dextran measurements of polarised NCI-N87 cells in co-culture conditions were performed after *H. pylori* infection for 24 h. The results were presented as a percentage of TEER or FITC-dextran transport compared to control (uninfected polarised NCI-N87 cells).

2.3. siRNA transfection

Cells were seeded at 2 × 10^5 cells on the porous membrane of the 12-well ThinCert™ inserts one day before transfection. Transfection of siRNA was performed using siLentFect™ (Bio-Rad, #1703362). Briefly, the cell culture media in both apical and basolateral compartments were changed to Opti-MEM (Gibco®/ Life Technologies) before transfection. siRNA against A20 (SI05018601, Qiagen) and scrambled siRNA (#D-001810-10, Dharmacon) were prepared at a final concentration of 50 nM and added onto the apical side. At 6 h after transfection, the medium was changed to a fresh RPMI 1640 medium containing 10% FCS. The cells were cultured for an additional 42 h before starting the co-culture experiment and infection.

2.4. Apoptotic cell death analysis

The cells were harvested using trypsin and stained with an Annexin V-FITC/PI Kit (MabTag GmbH), according to the manufacturer's instructions. Annexin V/PI stained cells were determined by flow cytometry using the CyFlow space (Sysmex). Ten thousand gated single cells were acquired and the data were processed and analysed using Flowing Software 2.5.1. The Annexin V-positive cells (early apoptotic cells) and Annexin V/PI double-positive cells (late apoptotic cells) were summed up to give the percentage of total apoptotic cells.

2.5. Preparation of cell lysates and immunoblotting

The cells on porous membrane were washed with ice-cold PBS, followed by lysis in RIPA lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 10 mM K_2HPO_4, 10% glycerol, 1% Triton X-100, 0.05% SDS) supplemented with 1 mM Na_3VO_4, 1 mM Na_2MoO_4, 20 mM NaF,

10 mM $Na_4P_2O_7$, 1 mM AEBSF, 20 mM Glycerol-2-phosphate, and 1× EDTA-free protease inhibitor mix (PI) (cOmplete™, Mini, Roche). Lysates were obtained after centrifugation (13,000 g, 4 °C, 10 min). The lysate samples were mixed with Laemmli buffer, heated at 95°C for 10 min, separated in SDS containing Tris-Glycine gels, and transferred onto PVDF membranes (Millipore). The membranes were blocked with 5% skim milk in TBS containing 0.1% Tween 20 (TBS-T) at room temperature (RT) for 1 h. The membranes were incubated with primary antibodies in either 5% BSA or 5% skim milk in TBS-T at 4 °C overnight and subsequently with appropriate HRP-conjugated secondary antibody in 5% skim milk in TBS-T at RT for 1 h. The immunoblot was then developed using a chemiluminescent substrate (#WBKLS0500, Millipore) and visualised using the ChemoCam Imager (Intas).

The following primary antibodies were used: A20 (sc-166692) was purchased from Santa Cruz Biotechnology; Caspase 3 (#9662), Caspase 8 (#9746), Cleaved Caspase 3 (#9661), Cleaved caspase 8 (#9496) and phospho-IκBα (#9246) were purchased from Cell Signaling Technology, and GAPDH (#MAB374) was purchased from Millipore.

2.6. Caspase-3/7 assay

The apical culture medium was replaced by a medium containing *H. pylori* (MOI 100) and Incucyte® Caspase-3/7 Green Dye (dilution 1:1000, Sartorius). At 24 h after *H. pylori* infection, the hanging arms of ThinCert™ insert were cut off and the insert was placed into a 12-well plate well. The plate was then placed into the Incucyte® S3 Live-Cell Analysis System (Sartorius) for measuring the fluorescence signal using the phase contrast and green fluorescence channel at a magnification of 20× (36 images per membrane). A set of nine images from the central region of the membrane were analysed by Image J software (National Institutes of Health) and presented as fluorescence intensity (A.U.).

2.7. Immunofluorescence

The mucosoid cultures on the porous membrane of the ThinCert™ inserts were fixed with 4% paraformaldehyde for 15 min, followed by three washes with PBS. The specimens were then blocked and permeabilised in PBS containing 1 %BSA (w/v), 2 % FCS (v/v) and 0.25 % (v/v) Triton-X-100 for 30 minutes at RT. The inserts were washed thrice with PBS, incubated with a primary antibody against E-cadherin (ab1416, Abcam) or occludin (611090, BD Biosciences) in 10x diluted blocking buffer at RT for 30 minutes. Afterwards washed thrice with PBS and incubated with the secondary antibody (AlexaFluor 488 or AlexaFluor 555, ThermoFisher Scientific) in 10x diluted blocking buffer at RT for 30 minutes, followed by three washes with PBS containing 0.1% (v/v) Tween-20. DAPI (100µg/ml, Sigma-Aldrich) diluted 1:60 in MilliQ water was added to stain the nuclei. In order to perform histology (H&E staining) and

immunofluorescence on filter sections the filters were fixed overnight in 4% paraformaldehyde at 4 °C, washed with PBS twice and embedded in Histogel (HG-4000-144) inside a cryomold (Tissue-Tek®). Further, the sample were processed overnight, paraffin embedded and 5 µm sections were cut with a rotation microtome (Leica) and mounted onto frosted slides. Deparaffinization, rehydration and H&E staining was performed according to standard protocols. Antigen was retrieved after rehydration by boiling the slides in citrate buffer pH 6.0 for 25 min. The immunofluorescence protocol was used as above. Images were acquired on an AxioObserver 7 equipped with the Colibri 5 RGB-UV, a camera Axiocam 305 color and a 40x objective (Zeiss Plan-Apochromat, NA 1.4, Oil a = 0.13 mm) using the software ZEN 3.0 pro (Carl Zeiss microscopy). The filter sets used were as follows: DAPI, 96 HE BFP; AlexaFluor 488, 38 HE GFP; and AlexaFluor 555, 43 HE DsRed.

2.8. Statistical analysis

Quantitative data were presented as mean ± SD (standard deviation) of at least two independent experiments. The statistical significance of data was analysed by applying Student's T-test. P-values ≤ 0.05, 0.01, 0.001 were considered significant (*, **, ***, respectively).

3. Results

3.1. Co-culture with fibroblasts protects the monolayer integrity of NCI-N87 cells during *H. pylori* infection

Gastric epithelial NCI-N87 cells were grown on the porous membrane of the ThinCert™ inserts to obtain a polarised cell monolayer. The cell monolayer's integrity was determined by measuring the transepithelial electrical resistance (TEER) and the amount of fluorescein isothiocyanate (FITC)-labelled dextran transported from the apical to the basolateral sides of the cell monolayer over time. An increase in TEER (Fig. 1A) and a decrease of FITC-dextran transport (Fig. 1B) was observed over time, in which the cells reached the state of the fully polarised monolayer with completed barrier integrity after 4 days of cell culture. Therefore, the polarised NCI-N87 cells at this time point were used for further experiments.

The interaction between epithelial cells and fibroblasts is closely linked to the gastric pathophysiology of bacterial infection [21]. Therefore, we established a co-culture system of polarised gastric epithelial NCI-N87 cells and human gastric fibroblasts (HGF) to simulate in part the gastric microenvironment (Fig. 1C), in which the polarised NCI-N87 cells were infected with *H. pylori* at the apical side and could communicate with HGF at the basolateral side. We then investigated the impact of HGF on the monolayer integrity of NCI-N87 cells upon *H. pylori* infection. We found a significant decrease in TEER (Fig. 1D) and an increase in FITC-dextran

transport (Fig. 1E) in polarised NCI-N87 cells after *H. pylori* infection, suggesting that *H. pylori* impairs the monolayer integrity of polarised NCI-N87 cells. Interestingly, the loss of TEER and increase in FITC-dextran passage was attenuated in the presence of HGF co-culture, but not if co-cultivated with NCI-N87 cells (Fig. 1D and E). Our results suggest a protective role of HGF on the monolayer integrity of the polarised NCI-N87 cells.

3.2. Co-culture with fibroblasts suppresses apoptotic cell death of NCI-N87 cells during *H. pylori* infection

We next investigated the impact of HGF on the apoptotic cell death of polarised NCI-N87 cells during *H. pylori* infection. Apoptotic cell death was determined by Annexin V/PI staining after NCI-N87 cells were infected with *H. pylori* for 24 h. We observed an increase in apoptotic cell death upon *H. pylori* infection (Fig. 2). Importantly, the number of apoptotic cells was significantly decreased in the presence of HGF co-culture, but not if co-cultivated with NCI-N87 cells. Quantitative analysis revealed that co-culture with HGF decreased apoptosis of polarised NCI-N87 cells by 68% at 24 h post-infection. However, this effect was not observed when NCI-N87 cells and NCI-N87 cells were co-cultured, indicating that co-culture specifically with HGF could suppress *H. pylori*-induced cell death in NCI-N87 cells.

3.3. Conditioned media from fibroblasts suppresses apoptotic cell death of NCI-N87 cells during *H. pylori* infection

Our co-culture system provides a platform where fibroblasts and polarised NCI-N87 cells can communicate through secreted factors in the medium. Therefore, we hypothesised that the HGF communicate with polarised NCI-N87 cells through secreted factors and thus protected NCI-N87 cells from death during *H. pylori* infection. We determined the impact of different conditioned media (CM) from HGF on *H. pylori*-induced cell death in polarised NCI-N87 cells. Briefly, three different conditioned media of HGF (CM of HGF mono-culture (yellow), CM of HGF in co-culture with non-infected polarised NCI-N87 cells (blue), and CM of HGF in co-culture with infected polarised NCI-N87 cells (green)) were collected and added to the basolateral side of the polarised NCI-N87 cells (Fig. 3). The polarised NCI-N87 cells were then infected with *H. pylori* for 24 h, after which apoptotic cell death of polarised NCI-N87 cells was analysed. We found that only the conditioned medium from HGF in co-culture with infected polarised NCI-N87 cells (green) was capable of decreasing *H. pylori*-induced apoptotic cell death in NCI-N87 cells, while the others had little or no effect. This result indicates that HGF contributes to the suppression of apoptotic cell death of polarised NCI-N87 via factors secreted in response to the presence of infected polarised NCI-N87 cells.

3.4. Co-culture with fibroblasts ameliorates *H. pylori*-induced A20 expression and cell survival of NCI-N87 cells

We have previously reported that *H. pylori* infection leads to activation of NF-κB signalling, which subsequently upregulates the NF-κB target gene *TNFAIP3,* encoding A20 [22]. A20 is a deubiquitinylase that can stabilise caspase-8, leading to the inhibition of caspase-8 activation and suppressed apoptotic cell death [22]. Therefore, we hypothesised that the co-cultivation of HGF cells might affect regulation of A20 and thereby cell survival in NCI-N87 cells. Interestingly, we observed an increase in *H. pylori*-induced IκBa phosphorylation and A20 expression, and a corresponding decrease in caspase-8 cleavage in NCI-N87 cells when co-cultured with HGF (Fig. 4A). Furthermore, depletion of A20 resulted in an increment of caspase-8 cleavage (Fig. 4A). Consistently, the IncuCyte® live-cell imaging analysis of the activated caspase-3/7 after 24 h of *H. pylori* infection showed a decrease in fluorescence intensity in NCI-N87 cells when co-cultured with HGF (Fig. 4B). Moreover, the fluorescence intensity increased upon A20 depletion (Fig. 4B).

So far, our results suggest the involvement of A20 in the suppression of apoptotic cell death in *H. pylori*-infected NCI-N87 cells during co-culture with HGF. A20 exerts its inhibitory effect via caspase-8, thus, we further examined whether inhibition of caspase-8 blocks apoptotic cell death in *H. pylori*-infected NCI-N87 cells co-cultured with HGF. Treatment with the caspase-8 inhibitor (Z-IETD-FMK) 15 min prior to *H. pylori* infection diminished apoptotic cell death of NCI-N87 cells (Fig. 5). However, the effect of caspase-8 inhibition was not more pronounced in NCI-N87 cells when co-cultured with HGF (Fig. 5). This indicates that co-culture with HGF suppresses apoptotic cell death of polarised NCI-N87 cells in a caspase-8 dependent manner.

3.5. Co-culture with fibroblasts suppresses apoptotic cell death of gastric mucosoids during *H. pylori* infection

To further corroborate our data, we used primary tissue and employed gastric mucosoid cultures [19]. Gastric mucosoids grown on a collagen-coated membrane as a highly polarised columnar epithelial layer with nuclei located on the basal side (Fig. 6A, left panel) were IF labelled for occludin to show the apical tight junction of the mucosoids, giving rise to a closed monolayer (Fig. 6A, middle panel). The infection of gastric mucosoids with GFP-labelled *H. pylori* was shown in Fig. 6A (right panel). Consistent with polarised NCI-N87 cells, we found that co-culture with HGF promoted *H. pylori*-induced IκBa phosphorylation and enhanced A20 expression in gastric mucosoids (Fig. 6B). Further, we also observed a decrease in caspase-8 cleavage in gastric mucosoids when co-cultured with HGF (Fig. 6B). Consistently, the IncuCyte® live-cell imaging analysis of the activated caspase-3/7 after 24 h of *H. pylori*

infection showed a decrease in fluorescence intensity in gastric mucosoids when co-cultured with HGF (Fig. 6C).

4. Discussion

Fibroblasts are the major stromal cells as part of the lamina propria of the gastric mucosa, that are closely in contact with the gastric epithelium and could affect gastric epithelial differentiation and proliferation [6]. Crosstalk between the stromal fibroblasts and epithelium is considered to act as a functional driver of gastric cancer development [23, 24]. However, little is known about the crosstalk between fibroblasts and gastric epithelium and its impact on epithelial cell survival. In this study, we performed experiments with *in vitro* co-culture systems by using either polarised human gastric cancer cell line NCI-N87 or gastric mucosoids in co-culture with HGF upon *H. pylori* infection. We showed that co-culture with HGF could suppress apoptotic cell death in polarised NCI-N87 cells during *H. pylori* infection. However, this effect was not observed in co-culture with NCI-N87 cells (Fig. 2), highlighting the significant role of gastric fibroblasts in suppressing apoptotic cell death in *H. pylori*-infected epithelial cells. The non-contact co-culture system used in this study indicated that the effect of co-cultivation of polarised NCI-N87 cells with HGF was initiated through factors secreted by HGF. Previously, keratinocyte growth factor (KGF) had been identified as the growth-stimulating factor from human gastric fibroblasts to human scirrhous gastric carcinoma cells [25]. Furthermore, Sun *et al*. [26] reported that fibroblast growth factor 9 (FGF9) is a novel growth factor overexpressed in cancer-associated fibroblasts and a possible secreted mediator that promotes the survival and invasive capability of gastric cancer cells. Notably, we observed that the conditioned medium of HGF after co-culturing with *H. pylori*-infected polarised NCI-N87 cells protected from apoptotic cell death (Fig. 3). Therefore, this suggests that the HGF respond to a component secreted basolaterally by polarised NCI-N87 cells after *H. pylori* infection.

Infection of *H. pylori* induces early and transient activation of NF-κB in gastric epithelial cells [27-29]. Dysregulation of NF-κB impacts on gastric inflammation and carcinogenesis due to the regulation of growth factors, anti-apoptotic factors and cytokine/chemokine production [30]. Our results showed a transient increase in phospho-IκBα, indicating NF-κB activity, and an increase in the expression of the NF-κB regulated molecule A20 in NCI-N87 cells infected with *H. pylori*, when co-cultured with HGF (Fig. 4A). Therefore, the crosstalk between the gastric epithelial cells and HGF suggest NF-κB dependency. A factor secreted from gastric epithelial cells, which induces a response in HGF, intensify the NF-κB activation in the gastric epithelial cells and subsequently promote cell survival. An impact of *H. pylori* infected-

epithelial cells on stroma cells has been reported by Ferrand *et al*. [31]. Here, it was shown that *H. pylori*-infected gastrointestinal epithelial cells secrete multiple cytokines, with a major role of TNF, mainly via the NF-κB-dependent pathway, which induces the migration of bone marrow-derived mesenchymal stromal cells to the site of infection [31]. In another study the secretome of activated gastric fibroblasts induced a cancer stem cell-related differentiation program in gastric epithelial cells, partially by TGFβ signalling [32]. Furthermore, the reciprocal interaction between gastric tumour cells and activated fibroblasts through TNF/IL-33/ST2L signalling has been reported, leading to a malignant phenotype [24].

Infection of *H. pylori* in gastric epithelial cells initiates moderate apoptotic cell death. The bacterial virulence factors such as vacuolating cytotoxin (VacA) and gamma-glutamyltranspeptidase (GGT) can trigger the intrinsic/mitochondria-dependent apoptotic pathway, while upregulation of TRAIL and FasL and their corresponding receptors upon *H. pylori* infection are implicated in triggering the extrinsic apoptotic pathway [9-17]. Caspase-8 is a master regulator of the extrinsic cell death pathway [33]. We previously reported that A20 deubiquitinylates caspase-8, suppressing efficient caspase-8 cleavage and apoptotic cell death [22]. Prominently, we found that co-culture with HGF strengthen increased A20 expression in *H. pylori*-infected NCI-N87 cells and suppressed the extent of caspase-8 cleavage in polarised NCI-N87 cells during *H. pylori* infection (Fig. 4A), indicating that enhanced expression of A20 via intensified NF-κB activation mediates the suppressive effect of HGF. This effect of A20 was antagonised by depletion of A20, resulting in an increase in caspase-8 and caspase-3 cleavage compared with the scrambled control. The treatment with a caspase-8 inhibitor abrogated caspase-8-dependent apoptotic cell death induced by *H. pylori* infection (Fig. 5) and demonstrate that the suppressive effect of HGF cells on apoptotic cell death of polarised NCI-N87 cells is caspase-8 dependent.

Moreover, we used human primary epithelial mucosoids, which recapitulate most of the functions of the human gastric epithelium [19]. In an air-liquid interface culture, the gastric mucosoids developed a continuous cell monolayer with columnar epithelial morphology of the gastric epithelium (Fig. 6A). The apical mucus secretion indicates for the gastric epithelial phenotype and constitutes a protective shield for the gastric mucosoids. Co-culture of gastric mucosoids and HGF represents a model of gastric epithelium-stroma communication. With this model, we could corroborate the effect of HGF co-culture on the protection of apoptotic cell death of gastric mucosoids *via* enhanced A20 expression during *H. pylori* infection (Fig. 6B and C).

5. Conclusion

Our data demonstrate the protective role of human gastric fibroblasts on apoptotic cell death of gastric epithelial cells. We identified enhanced expression of A20 regulated by NF-κB activity in response to *H. pylori* infection in gastric epithelial cells as an underlying mechanism for suppression of apoptotic cell death in gastric epithelial cells by HGF in co-culture. This can be attributed to a complex crosstalk via secreted factors between HGF and gastric epithelial cells. However, the identity of the secreted factor(s) involved in this crosstalk will need further investigation. Finally, our finding highlights the impact of cell-cell communication on cell survival in the gastric mucosa during *H. pylori* infection and might suggest potential therapeutic strategy by interfering with the interaction between fibroblasts and epithelial cells.

Credit authorship contribution statement

Phatcharida Jantaree: Investigation, Methodology, Writing-Original draft. **Yanfei Yu**: Investigation. **Supattra Chaithongyot**: Investigation. **Gunter Maubach**: Investigation, Writing-Review & Editing. **Christian Täger**: Investigation. **Mohsen Abdi Sarabi**: Investigation. ……. **Michael Naumann**: Conceptualization, Methodology, Writing & Editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

Data availability

All data generated and analysed during the current study are included in this article and its additional files.

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Figure legends

Fig. 1. Co-culture with fibroblasts protects the monolayer integrity of *H. pylori*-infected NCI-N87 cells. NCI-N87 cells were grown as a polarised cell monolayer on a porous membrane of ThinCert™ insert. (A) TEER values and (B) the percentage of FITC dextran were measured at the indicated time. (C) Schematic illustration of the mono- and co-culture system of polarised gastric epithelial cells (NCI-N87) and human gastric fibroblasts (HGF). *H. pylori* were added to the apical side of the polarised NCI-N87 cells. (D, E) Polarised NCI-N87 cells were infected with *H. pylori* in the absence or presence of either NCI-N87 cells or HGF co-culture for 24 h, followed by measurement of (D) TEER and (E) FITC dextran. TEER and FITC dextran were calculated as the percentage of polarized NCI-N87 control. Data information: Data shown in (A-B) and (D-E) are from two independent experiments and represent mean ± SD. *P≤0.05, ***P≤0.001 (Student's t-test).

Fig. 2. Co-culture with fibroblasts suppresses apoptotic cell death of polarized NCI-N87 cells during *H. pylori* infection. Polarised NCI-N87 cells were infected with *H. pylori* for 24 h in the absence or presence of either NCI-N87 cells or HGF co-culture. Polarised NCI-N87 cells were stained with Annexin V/PI, and the apoptotic cell death was analysed by flow cytometry. Data information: Data shown are representative of two independent experiments, and the graph represents mean ± SD from two independent experiments.

Fig. 3. Effect of fibroblast conditioned media on *H. pylori*-induced apoptotic cell death of polarised NCI-N87 cells. Conditioned media (CM) from HGF (from 3 different conditions) were collected including CM of HGF mono-culture (yellow), CM of HGF co-cultured with non-infected NCI-N87 cells (blue), and CM of HGF co-cultured with infected NCI-N87 cells (green). The conditioned medium was added to the basolateral side of the polarised NCI-N87 cells, infected with *H. pylori* for 24 h prior to staining with Annexin V/PI. The apoptotic cell death was analysed by flow cytometry. Data information: Data shown are representative of two independent experiments, and the graph represents mean ± SD from two independent experiments.

Fig. 4. Co-culture with fibroblasts ameliorates *H. pylori*-induced A20 expression and cell survival of NCI-N87 cells

(A) Polarised NCI-N87 cells were transfected with siRNA against A20 and infected with *H. pylori* in the absence or presence of HGF for indicated times. Whole-cell lysates were subjected to IB analysis of the indicated proteins. (B) Polarised NCI-N87 cells were transfected with siRNA against A20 and infected with *H. pylori* in the absence or presence of HGF for 24

h. Cleaved caspase-3/7 (green) in polarized NCI-N87 cells was detected by the IncuCyte® S3 Live-Cell imaging analysis system. Scale bars = 100 μm. Data information: Data shown in (A and B) are representative of two independent experiments. Fluorescence intensity in (B) depicts mean ± SD from two independent experiments. *P≤0.05, **P≤0.01 (Student's t-test).

Fig. 5. Co-culture with fibroblasts abrogates caspase-8-dependent apoptotic cell death in polarised NCI-N87 cells during *H. pylori* infection. Polarised NCI-N87 cells were pretreated with Z-IETD-FMK for 15 min, followed by *H. pylori* infection in the absence or presence of HGF for an additional 24 h. The cells were stained with Annexin V/PI, and apoptotic cell death was analyzed by flow cytometry. Data information: Data shown are representative of two independent experiments, and the graph represents mean ± SD from two independent experiments.

Fig. 6. Co-culture of gastric mucosoids with fibroblasts suppresses apoptotic death during *H. pylori* infection. (A) Gastric mucosoids grown on filter inserts were fixed, paraffin-embedded, cut into 5 μm sections and stained for H&E or E-cadherin by fluorescence (left). Whole mount filter inserts either uninfected or infected for 2 h with GFP-labelled *H. pylori* were IF stained for occludin. Scale bars = 20 μm (right). (B) Gastric mucosoids were infected with *H. pylori* in the absence or presence of HGF for indicated times. Whole-cell lysates were subjected to IB analysis of the indicated proteins. (C) Gastric mucosoids were infected with *H. pylori* in the absence or presence of HGF for 24 h. Cleaved caspase-3/7 (green) in the gastric mucosoids was detected by the IncuCyte® S3 Live-Cell imaging analysis system. Scale bars = 100 μm. Data information: Data shown in (B) are representative of three independent experiments. Data shown in (C) are representative of two independent experiments. Fluorescence intensity in (C) depicts mean ± SD from two independent experiments. *P≤0.05 (Student's t-test).

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Fig. 1.

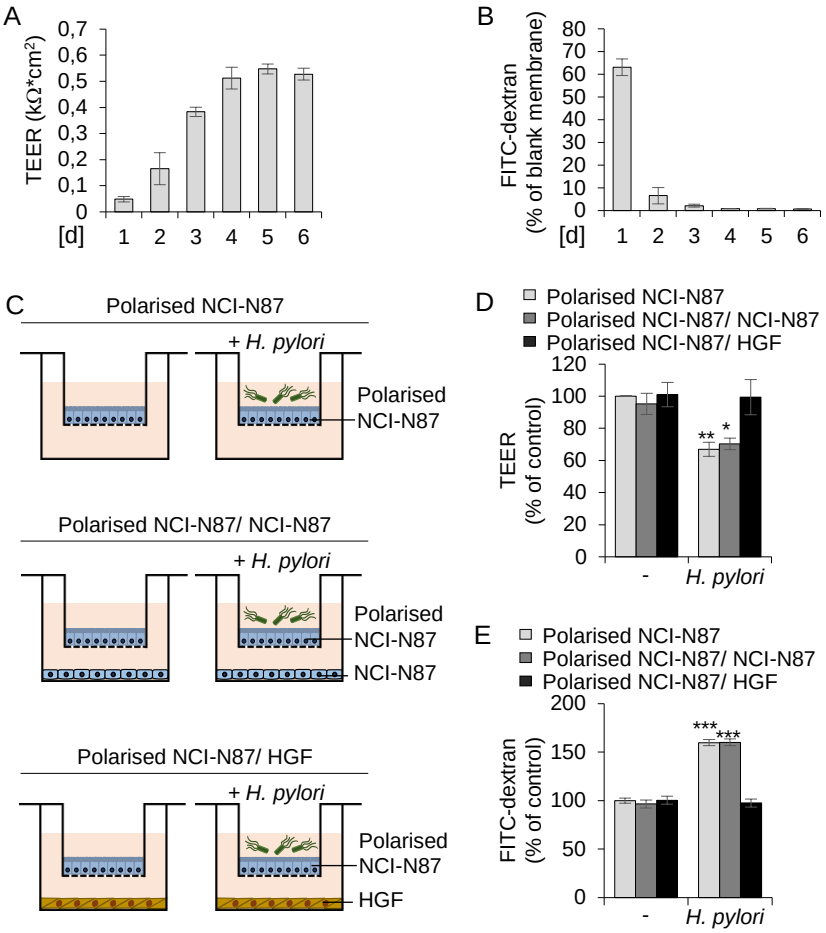

A
TEER (kΩ*cm²)
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
[d] 1 2 3 4 5 6
B
FITC-dextran (% of blank membrane)
80
70
60
50
40
30
20
10
0
[d] 1 2 3 4 5 6
C
Polarised NCI-N87
+ H. pylori
Polarised NCI-N87
Polarised NCI-N87/ NCI-N87
+ H. pylori
Polarised NCI-N87
NCI-N87
Polarised NCI-N87/ HGF
+ H. pylori
Polarised NCI-N87
HGF
D
Polarised NCI-N87
Polarised NCI-N87/ NCI-N87
Polarised NCI-N87/ HGF
TEER (% of control)
120
100
80
60
40
20
0
**
*
- H. pylori
E
Polarised NCI-N87
Polarised NCI-N87/ NCI-N87
Polarised NCI-N87/ HGF
FITC-dextran (% of control)
200
150
100
50
0
***
***
- H. pylori

Fig. 2.

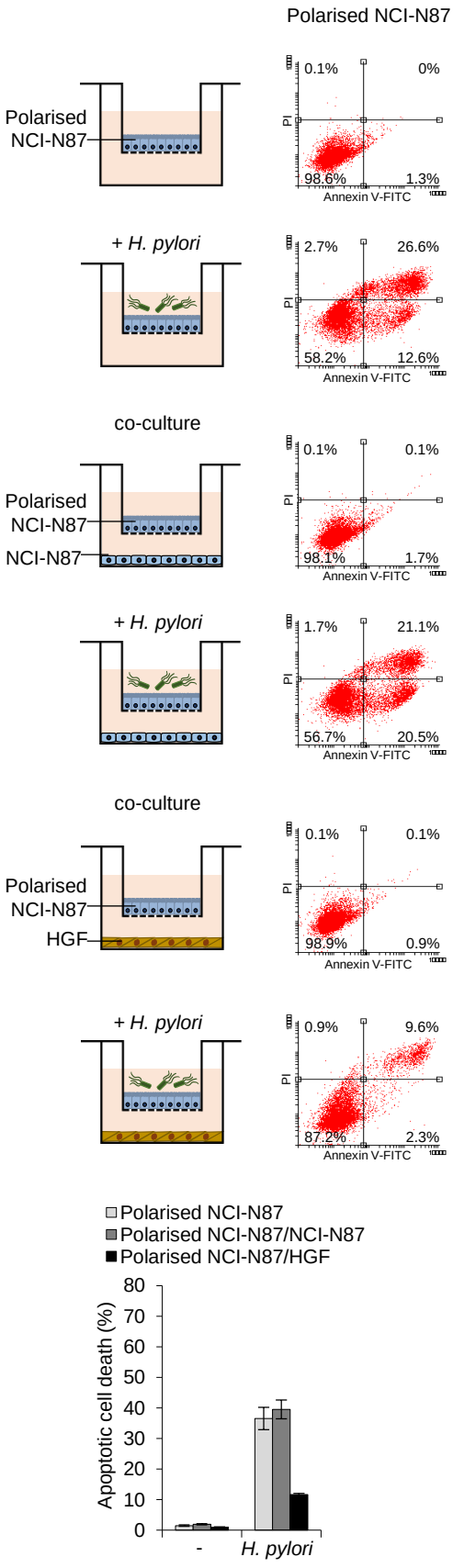
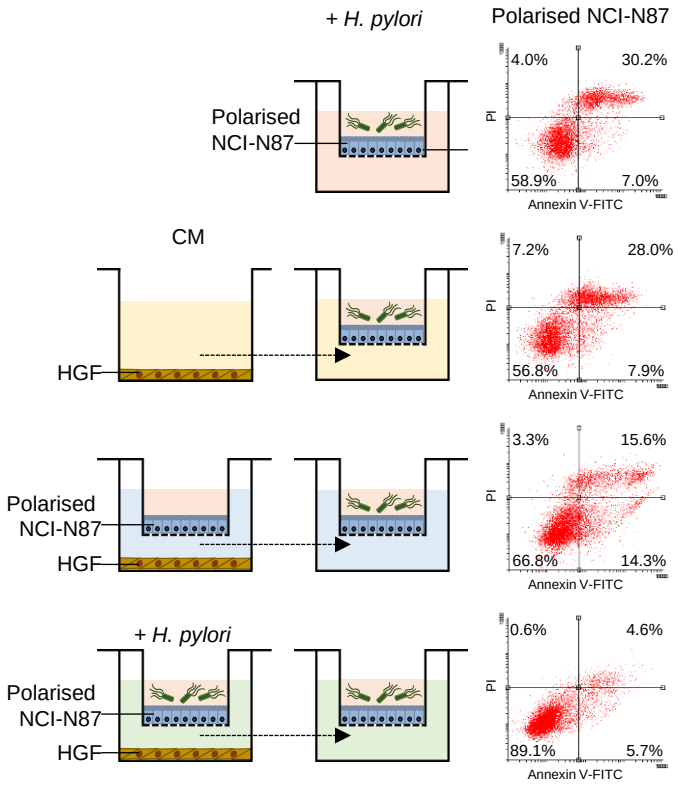
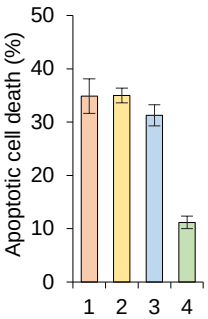

Polarised NCI-N87
Polarised NCI-N87
0.1%
0%
PI
98.6%
1.3%
Annexin V-FITC
+ H. pylori
2.7%
26.6%
58.2%
12.6%
co-culture
Polarised NCI-N87
NCI-N87
0.1%
0.1%
98.1%
1.7%
+ H. pylori
1.7%
21.1%
56.7%
20.5%
co-culture
Polarised NCI-N87
HGF
0.1%
0.1%
98.9%
0.9%
+ H. pylori
0.9%
9.6%
87.2%
2.3%
Polarised NCI-N87
Polarised NCI-N87/NCI-N87
Polarised NCI-N87/HGF
Apoptotic cell death (%)
80
70
60
50
40
30
20
10
0
-
H. pylori

Fig 3.





1: Control (RPMI)
2: CM HGF
3: CM HGF/ non-infected polarised NCI-N87 cells
4: CM HGF/ infected polarised NCI-N87 cells

Fig 4.

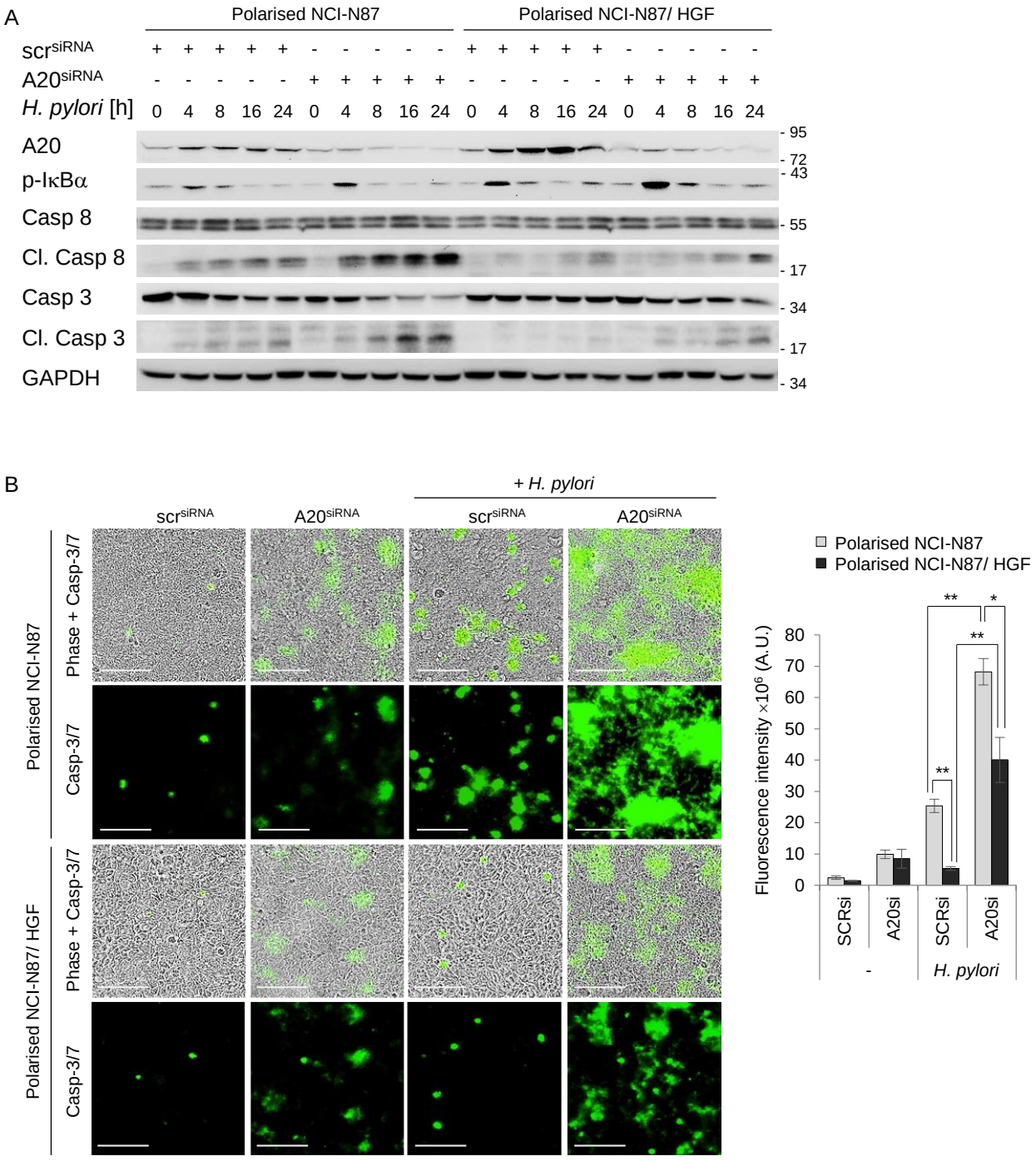

A
Polarised NCI-N87
Polarised NCI-N87/ HGF
scrsiRNA
A20siRNA
H. pylori [h]
0 4 8 16 24 0 4 8 16 24 0 4 8 16 24 0 4 8 16 24
A20
p-IκBα
Casp 8
Cl. Casp 8
Casp 3
Cl. Casp 3
GAPDH
95
72
43
55
17
34
17
34
B
+ H. pylori
scrsiRNA
A20siRNA
scrsiRNA
A20siRNA
Polarised NCI-N87
Polarised NCI-N87/ HGF
Phase + Casp-3/7
Casp-3/7
Phase + Casp-3/7
Casp-3/7
Polarised NCI-N87
Polarised NCI-N87/ HGF
Fluorescence intensity ×10⁶ (A.U.)
0 10 20 30 40 50 60 70 80
**
*
**
**
SCRsi
A20si
SCRsi
A20si
-
H. pylori

Fig 5.

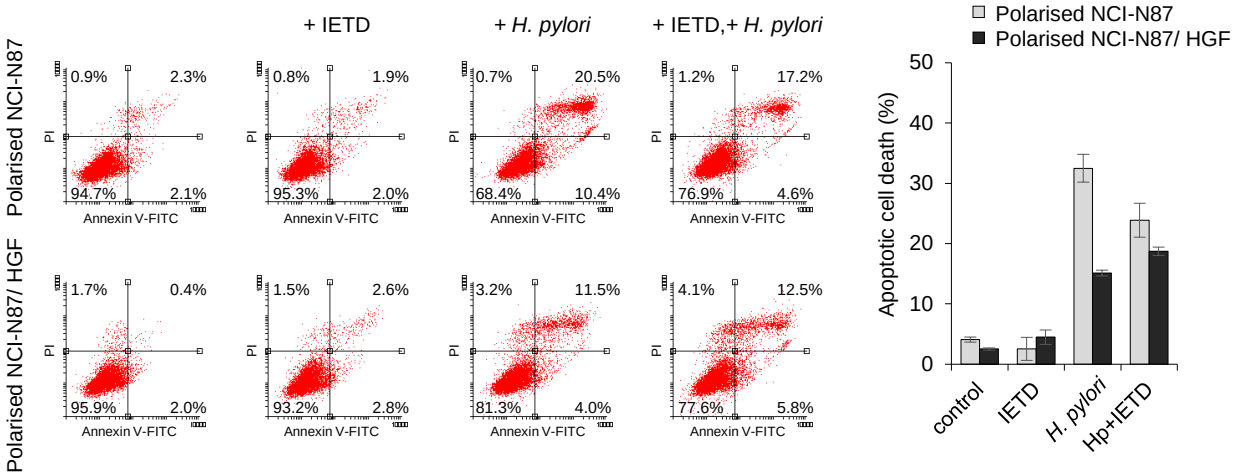
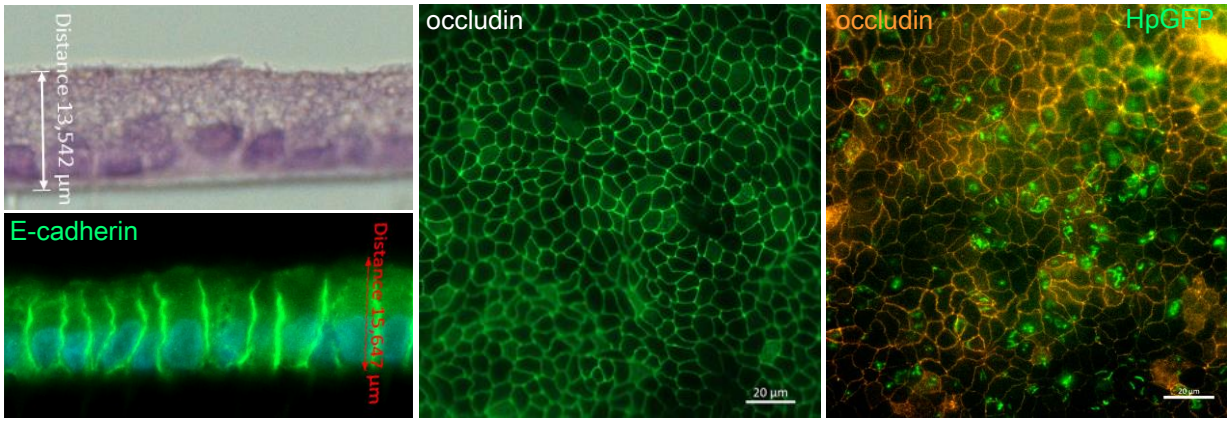
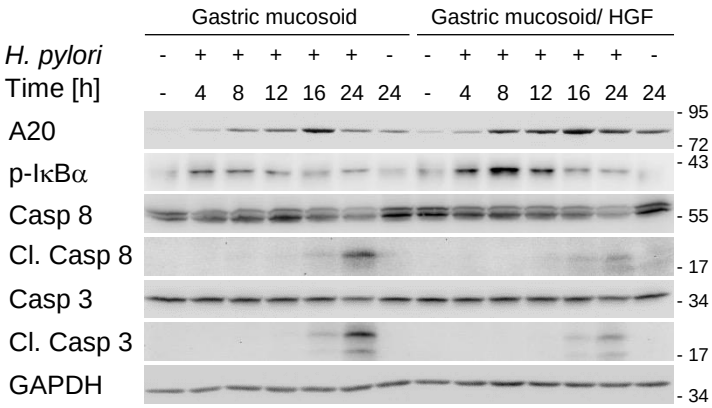
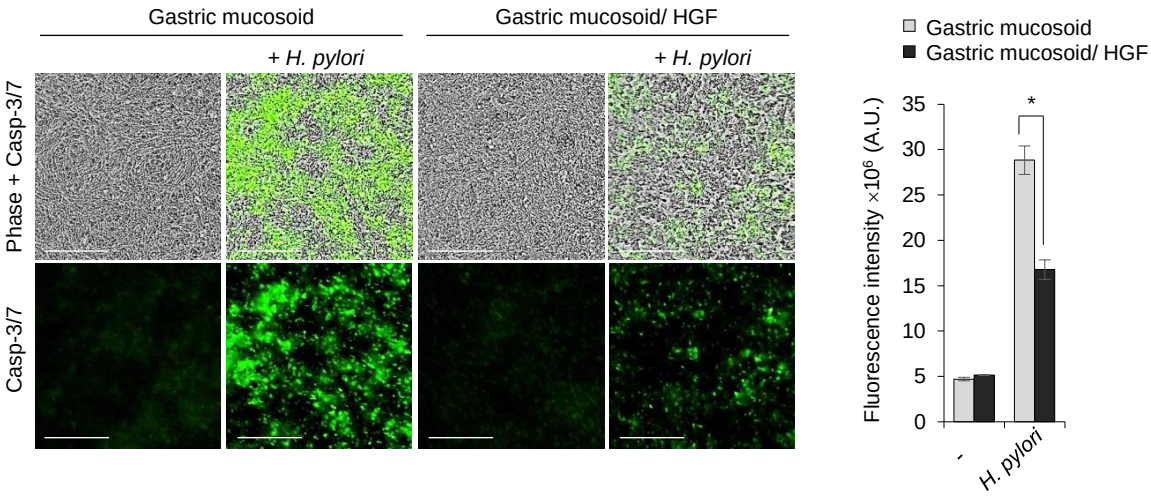

+ IETD
+ H. pylori
+ IETD,+ H. pylori
Polarised NCI-N87
Polarised NCI-N87/ HGF
PI
Annexin V-FITC
0.9%
2.3%
94.7%
2.1%
0.8%
1.9%
95.3%
2.0%
0.7%
20.5%
68.4%
10.4%
1.2%
17.2%
76.9%
4.6%
1.7%
0.4%
95.9%
2.0%
1.5%
2.6%
93.2%
2.8%
3.2%
11.5%
81.3%
4.0%
4.1%
12.5%
77.6%
5.8%
Polarised NCI-N87
Polarised NCI-N87/ HGF
Apoptotic cell death (%)
50
40
30
20
10
0
control
IETD
H. pylori
Hp+IETD

Fig 6.


A
Distance 13,542 µm
E-cadherin
Distance 13,542 µm
occludin
20 µm
occludin
HpGFP
20 µm


B
Gastric mucosoid
Gastric mucosoid/ HGF
H. pylori
- + + + + + - - + + + + + -
Time [h]
- 4 8 12 16 24 24 - 4 8 12 16 24 24
A20
p-IκBα
Casp 8
Cl. Casp 8
Casp 3
Cl. Casp 3
GAPDH
95
72
43
55
17
34
17
34


C
Gastric mucosoid
Gastric mucosoid/ HGF
+ H. pylori
+ H. pylori
Phase + Casp-3/7
Casp-3/7
Gastric mucosoid
Gastric mucosoid/ HGF
Fluorescence intensity ×10⁶ (A.U.)
35
30
25
20
15
10
5
0
*
-
H. pylori