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Research Article

miR-30c Increases the Intracellular Survival of *Helicobacter pylori* by Inhibiting Autophagy

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Persistent *Helicobacter pylori* infection causes a variety of gastrointestinal diseases and even gastric cancer. *H. pylori* invades gastric epithelial cells to survive and proliferate, which is one of the key factors in persistent colonization. A Published study has confirmed that cells can eliminate intracellular *H. pylori* through xenophagy to maintain intracellular balance. However, a growing body of evidences indicate that *H. pylori* can inhibit xenophagy by miRNA through regulating the expression of key autophagy-related genes. Through western blot analysis, mRFP-GFP-LC3 transfection assay, and transmission electron microscopy, we found that *H. pylori* infection obstructed autophagy flux degradation stage in GES-1 cell lines. Gentamicin protection assay confirmed that inhibit xenophagy is benefit for intracellular *H. pylori* survive. miR-30c-1-3p and miR-30c-5p were upregulated in GES-1 cell lines after infecting with *H. pylori*, resulting in the negative regulation on xenophagy. Further studies through bioinformatics analysis and dual-luciferase reporter assays confirmed that *ATG14* and *ULK1* were the target genes of miR-30c-1-3p and that *ATG12* was the target gene of miR-30c-5p. The overexpression of miR-30c-1-3p and miR-30c-5p reduces the expression of *ATG14*, *ULK1*, and *ATG12* at mRNA level and also decreased intracellular *H. pylori* elimination in GES-1 cells. The above results suggested that the inhibition on xenophagy by miR-30c-1-3p and miR-30c-5p through *ATG14*, *ULK1*, and *ATG12* targeting benefitted intracellular *H. pylori* in the evasion of xenophagy clearance.

1. Introduction

Helicobacter pylori, a Gram-negative spiral bacterium, can cause gastrointestinal diseases and even gastric cancer [1]. Approximately 50% of the world's population is infected with *H. pylori* [2]. The prevalence of *H. pylori* infection is lower in high-income countries than in low-income countries. Socioeconomic status was reported as one of the most important factors affecting the spreading of *H. pylori* infection [3]. *H. pylori* is an important factor that contributes to 65-80% of primary gastric cancer and has a strong correlation with gastric cancer [4]. Current first-line treatment for *H. pylori* includes triple therapy, nonbismuth quadruple therapy, and bismuth-containing quadruple therapy [5]. A growing body of evidence shows that *H. pylori* can intrude into in gastric epithelial cells [6, 7]. Intracellular *H. pylori* is responsible for treatment failure and persistent infection [8]. Studies have shown that increased doses of antibiotics are required to eliminate *H. pylori* that have invaded cells [8, 9]. The intracellular survival and proliferation of *H. pylori* are key factor of persistent infection.

Autophagy is the homeostasis mechanism that transports misfolded proteins and damaged organelles to lysosomes for degradation and reuse. It consists of four main stages: initiation, elongation, fusion, and degradation [10]. Studies have found that autophagy can also be used as an immune defense mechanism to help eliminate invading pathogens, and this mechanism was called xenophagy [8]. Xenophagy protects the body from a variety of pathogenic factors and plays a crucial part in antipathogen infection. Researchers have indicated that *H. pylori* can induce xenophagy in several gastric cell lines [11]. However, as bacteria

continue to adapt to the evolutionary process of xenophagy, new evidence has emerged showing that *H. pylori* has developed an escape mechanism against xenophagy [12]. This mechanism enables *H. pylori* to destroy the initiation and degradation processes of xenophagy, hijack autophagy vesicles for protection, proliferate in cells, and finally induce body damage [13]. Vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA) are two important virulence factors of *H. pylori*. With continued exposure to VacA disrupts the function of the cathepsin D enzyme and leads to the accumulation of incomplete autophagosomes. Besides, VacA was participated in the destruction of the autolysosome system [14]. Published studies have also shown that CagA is a negative regulator of autophagy that may inhibit autophagy and lead to P62 accumulation [15].

miRNAs offer information-rich values for disease diagnosis, and it can be used as biomarkers for disease diagnosis. Furthermore, a role for miRNAs in the pathogenesis of disease highlights their potential as mechanism-based targets for disease therapy [16]. miR-30 family is one of the earlier discovered miRNAs family. miR-30a inhibited Beclin-1 activity and thus prevented vesicle formation [17]. Tang and colleagues found that miR-30b directly targets *ATG12* and *BECN1* to inhibit xenophagy and promote the intracellular survival of *H. pylori*. These effects lead to the persistent infection and treatment failure in host cells [18]. miR-30d increases the intracellular survival of *H. pylori* through the inhibition of the xenophagy by targeting autophagy related genes [19]. Members of the same miRNA family may have part of the same base sequences and similar biological functions [20]. Whether miR-30c-1-3p and miR-30c-5p have regulatory effects on *H. pylori*-induced xenophagy needs further study.

In this research, we first examined the autophagy flux in GES-1 cells lines after *H. pylori* infection. Afterward, we examined whether miR-30c-1-3p and miR-30c-5p can regulate xenophagy function during *H. pylori* infection. We applied bioinformatic analysis and dual-luciferase reporter assays to further explore the potential target autophagy-related genes of miR-30c-1-3p and miR-30c-5p. Finally, we evaluated the effect of miR-30c-1-3p and miR-30c-5p on intracellular *H. pylori* by performing gentamicin protection assays and immunofluorescence microscopy.

2. Method

2.1. Chemicals and Reagents. DMEM and fetal bovine serum (FBS) were obtained from Gibco, USA. Sheep blood was procured from Pingrui Biotechnology, China. Columbia agar base was obtained from OXOID, USA. Saponin was bought from Sigma Aldrich, USA. Gentamicin sulfate was provided by Toku-E, Japan. 3-Methyladenine (3-MA), chloroquine (CQ), and rapamycin were acquired from Meilun Biotechnology, China. Bafilomycin A1 was purchased from APEx Biotechnology, China. The riboFECT CP transfection reagent was obtained from Ribobio, China.

2.2. Antibodies and Plasmids. Antilight chain 3 A/B (12741S), anti-P62 (39749S), and anti-rabbit IgG HRP-linked antibody (7074S) were obtained from Cell Signaling Technology Biotechnology, USA. Anti-*H. pylori* (ab20459) was provided by Abcam, UK. Antibodies against ACTIN (1338) were procured from Signalway Antibody, USA. Alexa Fluor 488 (101030) was acquired from Southern Biotech, USA. Hoechst 33342 solution was bought from Thermo Scientific, USA. The HBAD-mRFP-GFP-LC3 adenovirus, the mimic and inhibitor of miR-30c-1-3p, the mimic and inhibitor of miR-30c-5p, negative mimic, and negative inhibitor were sourced from Hanbio Biotechnology, China. Dual-Luciferase Reporter Assay System was obtained from Promega, USA.

2.3. H. pylori Strains. The *H. pylori* strain ATCC43504 was purchased from the American Type Culture Collection. *H. pylori* was cultured on Columbia agar base containing 5% sheep blood at 37°C in a tri-gas incubator (Nuaire Nu-5831E, USA). After 48–72 h, *H. pylori* was scraped and passaged on fresh Columbia blood agar. Turbidimetry (Zhuhai BASO Biotechnology Co. Ltd.) was utilized to adjust the McFarland standard of *H. pylori* strain if needed.

2.4. Cell Culture and Grouping. GES-1 was obtained from BeNa Culture Collection, China. HEK-293T was obtained from National Collection of Authenticated Cell Cultures, China. The cells were cultured in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 humidified incubator (CO150, USA). The cells were passaged when the cells reached 80% density.

Baf A1 (10 nM for 2 h), autophagy inhibitors (2 mM 3-MA for 12 h, or 10 μM CQ for 30 min), and autophagy inducers (serum-free starvation culture for 4 h or 200 nM rapamycin for 12 h) were selected in some assays. In brief, after pretreating with different drugs, the cells were cocultured with or without *H. pylori* at the multiplicity of infection (MOI) of 100 : 1. The protein expression or the quantity of intracellular *H. pylori* was observed.

For the transfection assay, the cells were cultured in six-well plates at a density of 0.4×10^6 cells/well. The cells were transfected with 100 nM mimic or inhibitor of miR-30c-1-3p and miR-30c-5p by using riboFECT CP transfection reagent and then incubating in medium at 37°C in 5% CO_2 for 36 h. The negative mimic and negative inhibitor served as the control. Afterward, the cells were cocultured with or without *H. pylori* for 12 h at the MOI of 100 : 1.

2.5. Western Blot Analysis. The cells were lysed in radio immunoprecipitation assay (RIPA) buffer containing protease inhibitors (CWBIO, China) in ice. Subsequently, the samples were collected, sonicated, and centrifuged at 4°C and 10 000 × *g* for 10 min. The supernatant was collected, and protein concentration was measured by using a BCA protein assay kit (Beyotime Biotechnology, China). After concentration equilibration to 2 μg/μl with RIPA, the samples were mixed with loading buffer and boiled at 100°C for 5 min. Equal amounts of protein samples were loaded onto SDS-PAGE polyacrylamide gels (4%–20% [*w*/*v*]). After electrophoresis at 80 V for 10 min and 120 V for 80 min, the proteins were transferred onto a polyvinyllidene fluoride membrane (Merck Millipore, USA). The membrane was washed three times with TBS

Table 1: Primer sequences for RT-qPCR.

Gene	Gene Forward primer (5′-3′)	Reverse primer (5′-3′)
ATG14	TGTACCTGGTCAGTCCAAGCTC	CAGGTCGGTTTCTTCATCGCTG
ULK1	GCAAGGACTCTTCCTGTGACAC	CCACTGCACATCAGGCTGTCTG
ATG12	AGTAGAGCGAACACGAACCATCC	AAGGAGCAAAGGACTGATTCACATA
GADPH	CCACATCGCT CAGACACCAT	GGCAACAATATCCACTTTACCAGAGT

containing 0.5% Tween-20 (v/v) and blocked with 5% nonfat milk for 2 h at room temperature. The membrane was then washed with TBST three times and incubated overnight at 4°C with anti-LC3 (1 : 1000, CST), anti-P62 (1 : 1000, CST), or anti-ACTIN (1 : 5000, SAB). After being washed with TBST three times, the membrane was further incubated with secondary antibodies for 2 h at 1 : 5000 dilution (Cell Signaling Technology Biotechnology). Finally, the blots were visualized by using ECL reagent and a 5200 chemiluminescence apparatus (Tanon). The results were analyzed by using ImageJ.

2.6. Quantitative RT-PCR. Total RNA was extracted by using the RNApure Tissue and Cell Kit following the instructions (CWBIO, China). RNA concentration was measured spectrophotometrically at 260 nm, and the concentration of RNA used in the study was more than 200 ng/μl. The absorbance ratio of 260/280 nm was used to measurement RNA quality, and samples with A260/A280 between 1.9 and 2.1 were considered to be of good quality. Total RNA was reverse transcribed into cDNA by using a First-Strand cDNA kit (TIANGEN, China), and quantitative RT-PCR was performed in accordance with the instructions included with the fluorescence quantitative detection kit (TIANGEN, China). The primer sequences are shown in Table 1. *GADPH* was utilized as the reference gene. Quantitative RT-PCR analyses for the mRNAs of *ULK1*, *ATG14*, and *ATG12* were performed by using a CFX96 real-time fluorescence quantitative PCR (RT-qPCR) instrument (Bio-Rad, USA). The RT-qPCR parameters were set as follows: predenaturation at 95°C for 15 min followed by 40 cycles at denaturation at 95°C for 10 s and annealing at 60°C for 20 s. The results were quantified through the Pfaffi method.

miRNA was extracted by using a miRNA purification kit following the instructions (CWBIO, China), and the concentration of miRNA used in the study was more than 200 ng/μl. A miRNA First-Strand cDNA kit (TIANGEN, China) was used to prepare cDNA. miRcute Plus miRNA qPCR kits (TIANGEN, China) were used for the RT-qPCR to quantify the expression miR-30c-5p and miR-30c-1-3p. U6 was utilized as the reference gene. The RT-qPCR parameters were set as follows: at 95°C for 15 min followed by 40 cycles at 94°C for 20 s and at 60°C for 34 s. Relative expression was quantified by using the $2^{-\triangle\triangle CT}$ method.

2.7. Gentamicin Protection Assay. After coculturing with *H. pylori* at the MOI of 100 : 1, PBS was used to remove nonadherent bacteria. For the determination of intracellular *H. pylori* number, GES-1 cell monolayers were treated with gentamicin (100 μg/ml; Toku-E, Japan) in the cell incubator for 6 h and then incubated with 250 μl of 0.5% saponin (Sigma, USA) at 37°C for 15 min. After diluting, plated on Columbia blood agar. The number of colonies formed 5 days later was counted as the intracellular bacterial number.

2.8. Immunofluorescence Microscopy. After infection by *H. pylori*, extracellular bacteria were clear through incubation with gentamicin (100 μg/ml) for 6 h. Adhesive cells were fixed in 4% paraformaldehyde (Meilun Biotechnology, China) for 10 min, permeabilized with 0.1% Triton X-100 (Coolaber Biotechnology, China) for 30 min, and blocked with 5% donkey serum in PBS for 1 h. The cells were then incubated with anti-*H. pylori* (Abcam; UK) overnight at 4°C. On the next day, the samples were washed three times. The cells were then incubated with Alexa Fluor488-conjugated goat anti-rabbit (Southern Biotech, USA) for 2 h at room temperature. Finally, the samples were stained with Hoechst 33342 (Thermo Scientific, USA) for 15 min and observed by using a LSM 800 confocal microscope (Zeiss). Data analyzed was by using ImageJ software.

2.9. mRFP-GFP-LC3 Adenovirus Transfection Assay. The cells were transfected with HBAD-mRFP-GFP-LC3 (Hanbio Biotechnology, China) at 6.3×10^7 PFU/ml for 24 h at 37°C in 5% CO_2. Afterward, cocultured with at the MOI of 100 : 1 for 12 h. The samples were fixed in 4% paraformaldehyde and then observed by using a LSM 800 confocal microscope (Zeiss). Yellow dots indicated autophagosomes, and free red dots indicated autolysosomes. Colocalization was analyzed by applying ImageJ software.

2.10. Transmission Electron Microscopy. The cells were mixed with electron microscope fixed solution (Wuhan Servicebio technology CO., LTD, China) and collected, then wrapped in 1% agarose and washed with 0.1 M phosphate buffer (pH 7.4) three times for 15 min each time. The cells were post-fixed with 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature and washed with 0.1 M phosphate buffer (pH 7.4) three times for 15 min each time. The samples were dehydrated with a graded alcohol and acetone series for 15 min each time. They were then permeabilized with acetone and 812 embedding media. The samples were inserted into an embedding plate and then baked overnight at 37°C. After polymerization at 60°C for 48 h, 60–80 nm ultrathin sections were cut on a ultramicrotome (Leica; Germany), counterstained with 2% uranyl acetate and lead citrate, dried at room temperature overnight, and examined under a HITACHI transmission electron microscope.

2.11. Bioinformatics and Dual-Luciferase Reporter Assay. TargetScan7.2 (http://www.targetscan.org/vert_72/) and miRDB (http://mirdb.org/) were used to predict the potential target

genes and the binding site for miR-30c-1-3p and miR-30c-5p. HEK-293T cells were plated on 96-well plates at 50–70% confluence. The cells were transfected with 0.16 μg of the luciferase reporter vector and 5 pmol miRNA mimic or negative mimic using the transfection reagent (Hanbio Biotechnology, China) at 37°C in 5% CO_2 for 6 h and subsequently exchanged for fresh culture medium. After 48 h of transfection, the cells were analyzed for luciferase activity by using a dual-luciferase assay system (Promega, USA). The Firefly luciferase value as the internal reference value, *Renilla* luciferase value as the reporter value. The relative ratio (Firefly/*Renilla*) was calculated. For each transfection, the luciferase activity assay was performed with three replicates.

2.12. Statistical Analysis. Statistical analysis was performed with SPSS 20.0, and the data were presented as $\bar{x} \pm$ sem. Differences between two groups were tested by Student's t-test. Differences among multiple groups were analyzed through one-way ANOVA followed by Dunnett's test or the LSD test to compare differences among groups. A difference was considered statistically significant when $P < 0.05$.

3. Results

3.1. H. pylori Infection Blocked the Degradation Stage of Autophagy Flux in GES-1 Cell Line. H. pylori infection promoted the expression of LC3B-II in a time-dependent manner ($P < 0.05$). However, no obvious alteration in P62 protein levels was observed upon *H. pylori* infection (Figure 1(a)). Furthermore, compared to the control group, Baf A1 challenge leads to the accumulation of LC3B-II and P62 in GES-1 cells after 6 and 12 h of infection with *H. pylori* ($P < 0.05$), suggested that *H. pylori* infection induced cellular xenophagy but inhibited autophagy flux (Figure 1(b)). Similar results were obtained based on the GFP-mRFP-LC3 transfection assay. The number of autophagosomes (yellow puncta) and autolysosome (free red puncta) increased after *H. pylori* infection after 12 h of infection relative to noninfected GES-1 cells ($P < 0.05$) (Figure 1(c)). Taken together, these data indicated that *H. pylori* infection induced autophagy flux but autophagy flux degradation stage was blocked in GES-1 cell lines.

3.2. Inhibition of Xenophagy Helps H. pylori Survive in Cells. The presence of autophagosomes (red arrowheads) and autolysosomes (blue arrowheads) in *H. pylori*-infected cells was observed by TEM (Figure 2(a)). The TEM results also showed that *H. pylori* infection induced visible cell membrane damage (black arrowheads) (Figure 2(a)). After autophagy inhibition (3-MA or CQ), the number of intracellular *H. pylori* increased significantly in GES-1 cells ($P < 0.01$). The opposite effect was observed under pretreatment with an autophagy inducer (starvation or rapamycin, $P < 0.05$). These data indicated that the inhibition of xenophagy contributed to the intracellular survival of *H. pylori* in GES-1 cells (Figures 2(b) and 2(c)).

3.3. miR-30c-1-3p and miR-30c-5p Played a Negative Regulatory Role in Xenophagy Induced by H. pylori Infection. The result of RT-qPCR revealed that the expression of miR-30c-1-3p (approximately 5-fold increment in 24 h) and miR-30c-5p rapidly increased from 3 h to 24 h after infection with *H. pylori* in GES-1 cell lines ($P < 0.05$) (Figure 3(a)).

The results of western blot revealed that compared with the negative inhibitor, the miR-30c-1-3p inhibitor significantly promoted LC3B-II conversion and P62 degradation ($P < 0.05$). The miR-30c-5p mimic significantly reduced the expression of LC3B-II, which was promoted by the miR-30c-5p inhibitor ($P < 0.05$). However, miR-30c-5p had no effect on P62 protein expression (Figure 3(b)). The TEM images demonstrated that compared with the negative mimic group, GES-1 cells transfected with the mimics (miR-30c-1-3p and miR-30c-5p) showed reductions in the quantity of autophagosomes and autolysosomes after 12 h of stimulation with *H. pylori.* By contrast, GES-1 cells transfected with the inhibitors (miR-30c-1-3p and miR-30c-5p) presented more autophagosomes and autolysosomes than the negative inhibitor group ($P < 0.05$) (Figure 3(c)).

The results of adenovirus transfection assay showed that the miR-30c-1-3p and miR-30c-5p inhibitors significantly increased the number of autophagosomes and autolysosomes in GES-1 cells relative to that in the negative inhibitor group ($P < 0.05$). Nevertheless, the miR-30c-1-3p and miR-30c-5p mimics significantly decreased the amount of autophagosomes and autolysosomes compared with the negative mimic ($P < 0.05$) (Figure 3(d)). These data indicated that miR-30c-1-3p and miR-30c-5p played a negative regulatory role in xenophagy induced by *H. pylori* infection.

3.4. miR-30c-1-3p and miR-30c-5p Reduce the Expression of Multiple Key Autophagy-Related Genes at mRNA Level. Screening for miR-30c-1-3p and miR-30c-1-5p targets revealed that the potential binding site for miR-30c-1-3p was the 3′ untranslated region (UTR) of *ATG14* and *ULK1*, whereas the 3′-UTR of *ATG12* was the potential binding site of miR-30c-5p (Figure 4(a)). The dual-luciferase reporter assay revealed that miR-30c-1-3p mimic significantly decreased luciferase activity in HEK-293T cells transfected with ATG14-WT and ULK1-WT reporter plasmids ($P < 0.001$), whereas no significant changes were observed in the cells transfected with mutant reporter plasmid. Moreover, miR-30c-5p significantly decreased luciferase activity in HEK-293T cells transfected with the ATG12-WT reporter plasmid ($P < 0.001$), whereas no significant changes were observed in the cells transfected with mutant reporter plasmid (Figure 4(b)). The results of the dual-luciferase reporter assay suggested that *ATG14* and *ULK1* were the target genes of miR-30c-1-3p and that *ATG12* was the target gene of miR-30c-5p.

RT-qPCR results demonstrated the mRNA levels of *ATG14* and *ULK1* were remarkably suppressed in GES-1 cells transfected with miR-30c-1-3p mimics compared with those in GES-1 cells transfected with the negative mimic ($P < 0.05$) but were increased in cells transfected with the miR-30c-1-3p inhibitor relative to those in cells transfected with the negative inhibitor ($P < 0.05$). The miR-30c-5p mimic decreased the expression level of *ATG12* compared with the negative mimic ($P < 0.01$), whereas *ATG12* expression in the miR-30c-5p inhibitor group was significantly higher than that in the negative inhibitor group ($P < 0.05$)

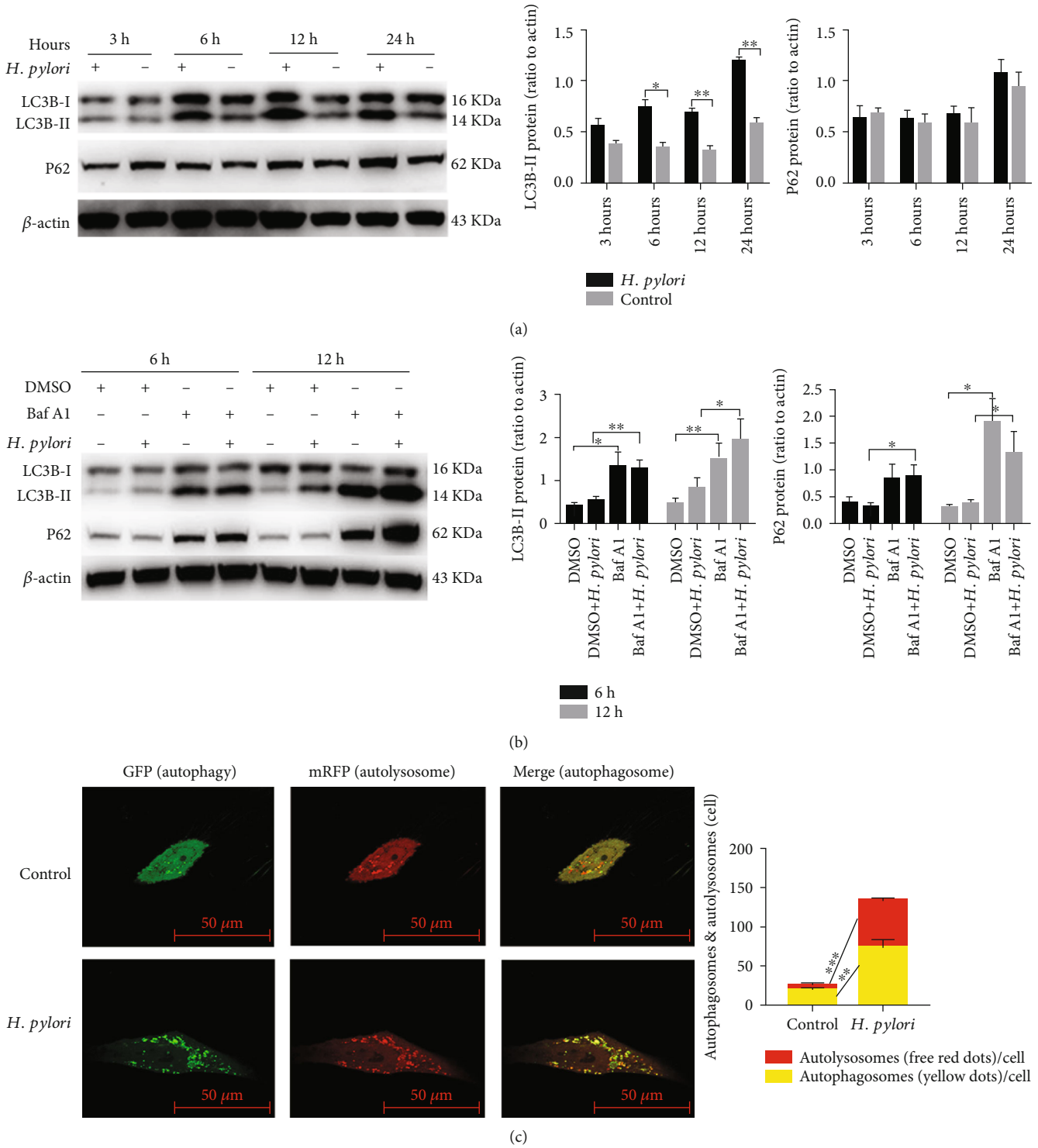


FIGURE 1: *H. pylori* infection induced incomplete autophagy flux in GES-1 cell lines. (a) GES-1 cells were infected with *H. pylori* (MOI = 100 : 1) for different times. *H. pylori* infection increases the expression of LC3B-II in time-dependent manner ($n = 3$). But no obvious alteration of P62 protein level was observed ($n = 3$). (b) The GES-1 cells were pretreated with Baf A 1 (10 nM) and then stimulated with *H. pylori* (MOI = 100 : 1) ($n = 5$). (c) After GES-1 cells were transfected with mRFP-GFP-LC3 adenovirus for 24 h, the cells were stimulated with *H. pylori* for another 12 h. The number of puncta in each cell was counted. *H. pylori*-infected GES-1 cells can significantly increase the number of autophagosomes and autolysosomes ($n = 4$). All error bars indicate SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

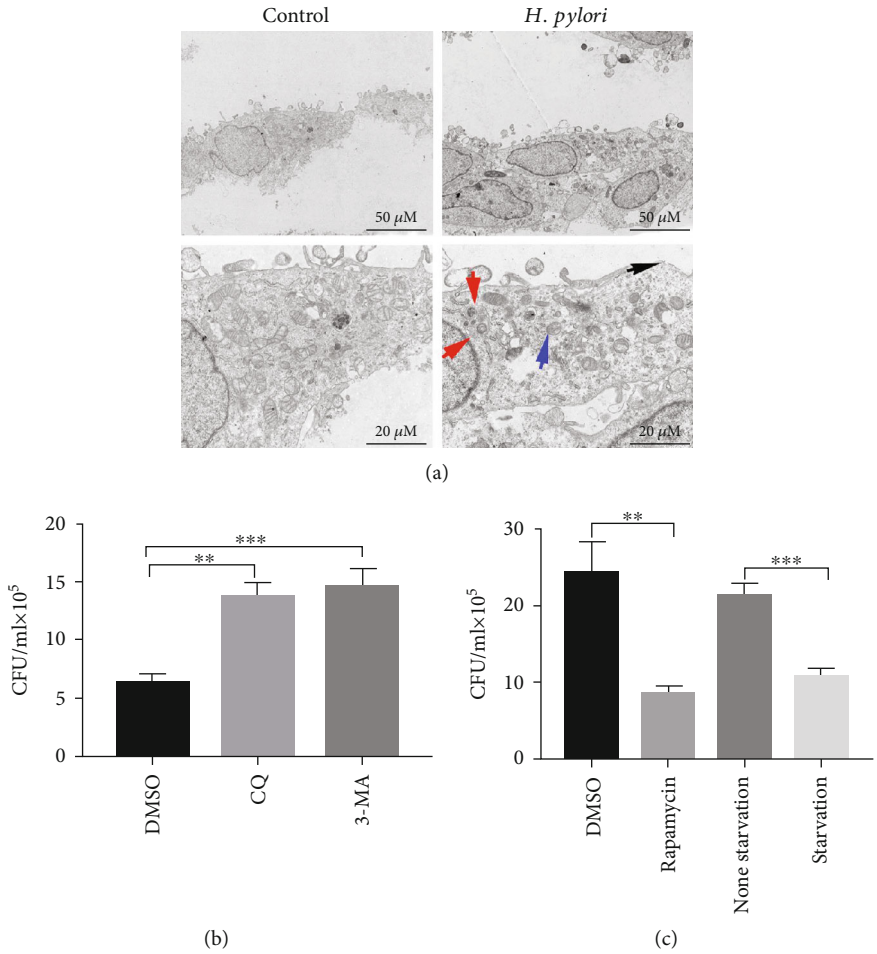


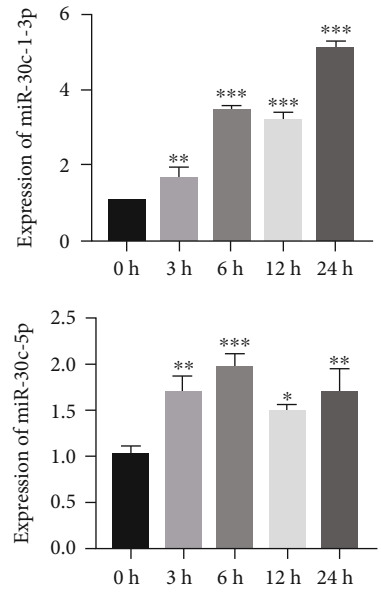
Figure 2: Inhibition of xenophagy can increase *H. pylori* survival in GES-1 cells. (a) Representative TEM picture of GES-1 cells after 12 h infection with *H. pylori*. Red arrows indicate autophagosomes, blue arrows indicate autolysosomes, and black arrows indicate damaged membrane. (b, c) The effects of xenophagy on the *H. pylori* survival in GES-1 cells. After preprocessing by starvation, DMSO, rapamycin (200 nM), 3-MA (2 mM), or CQ (10 μM), GES-1 cells were infected with *H. pylori* for 12 h and then lysed. Intracellular bacteria were quantified by using gentamycin protection assay ($n = 6$). All error bars indicate SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

(Figure 4(c)). These results suggested that miR-30c-1-3p and miR-30c-5p regulated autophagy-related genes at the mRNA level through target mRNA degradation.

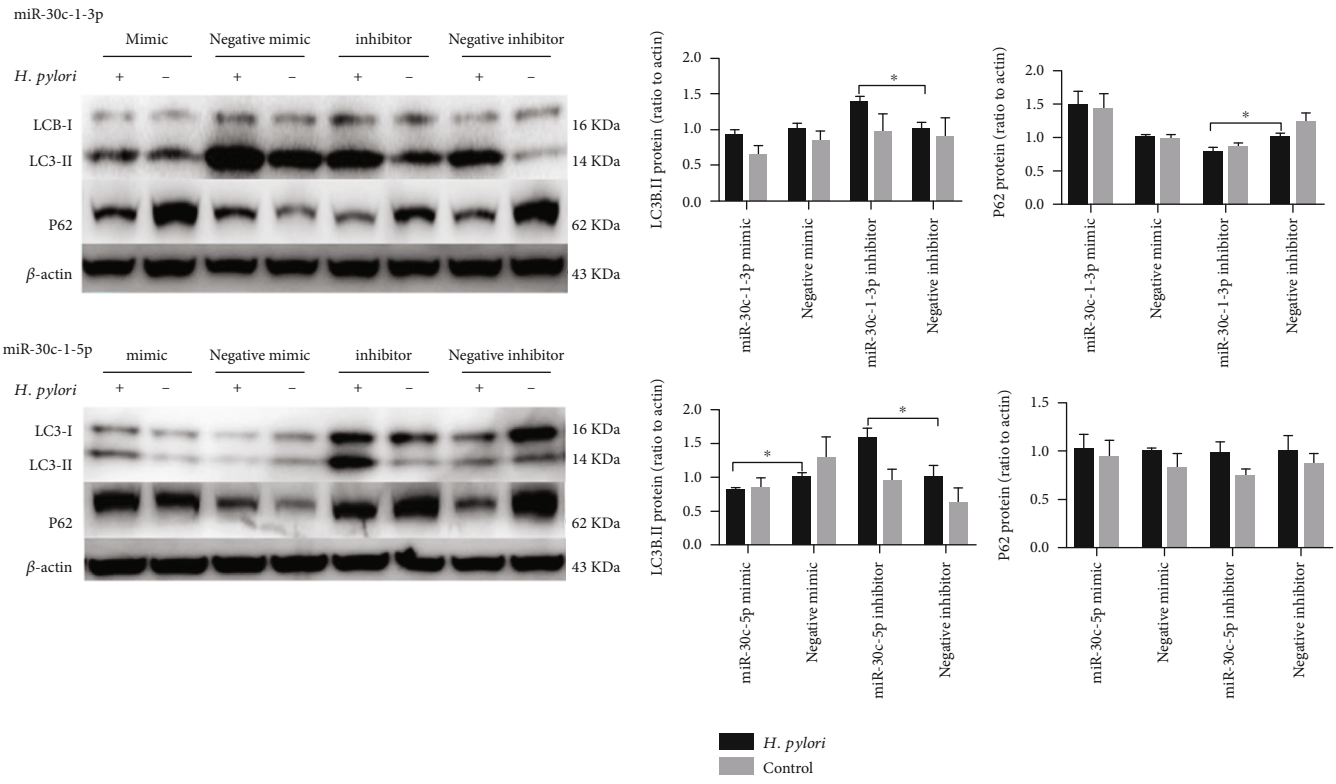
3.5. miR-30c-1-3p and miR-30c-5p Decrease Intracellular H. pylori Elimination in GES-1 Cells. The immunofluorescence microscopy experiment showed that the quantity of endocellular *H. pylori* cells was increased in the miRNA mimic (miR-30c-1-3p and miR-30c-5p) group than in the negative mimic group but was lower in the miRNA inhibitor group than in the negative inhibitor group ($P < 0.05$) (Figures 5(a) and 5(b)). The gentamicin protection assay revealed that the number of colonies in the miR-30c-1-3p group had increased by approximately 1.5-fold compared with that in the negative mimic group ($P < 0.05$) (Figure 5(c)). The number of colonies in the miR-30c-5p mimic group had increased by approximately 2.5-fold compared with that negative mimic group, whereas that in the miR-30c-5p mimic group had decreased by half ($P < 0.05$) (Figure 5(d)). Together, these dates suggested that miR-30c-1-3p and miR-30c-5p reduced intracellular *H. pylori* elimination from GES-1 cells.

4. Discussion

H. pylori is one of the important factors for a series of gastric diseases, including chronic gastritis and peptic ulcer. Persistent *H. pylori* infection can increase the risk of intestinal metaplasia and gastric cancer [21]. Although *H. pylori* was previously regarded as an extracellular bacterium, growing evidence has reported the facultative intracellular nature of *H. pylori*. Studies have shown that the body cells can eliminate intracellular bacteria through the xenophagy [22]. However, *H. pylori* establishes an escape mechanism against xenophagy and even hijacks autophagy vesicles for protection, proliferates in cells, and finally achieves persistent infection [13]. Numerous pieces of evidence from studies have revealed that miRNAs can affect xenophagy in many infectious processes by targeting autophagy-related genes [23]. In this research, we confirmed that *H. pylori* infection upregulates the expression of miR-30c-1-3p and miR-30c-5p in GES-1 cell. This effect inhibited the expression of autophagy-related genes and then suppressed the effect of xenophagy and eventually led to the persistent survival of intracellular *H. pylori*.

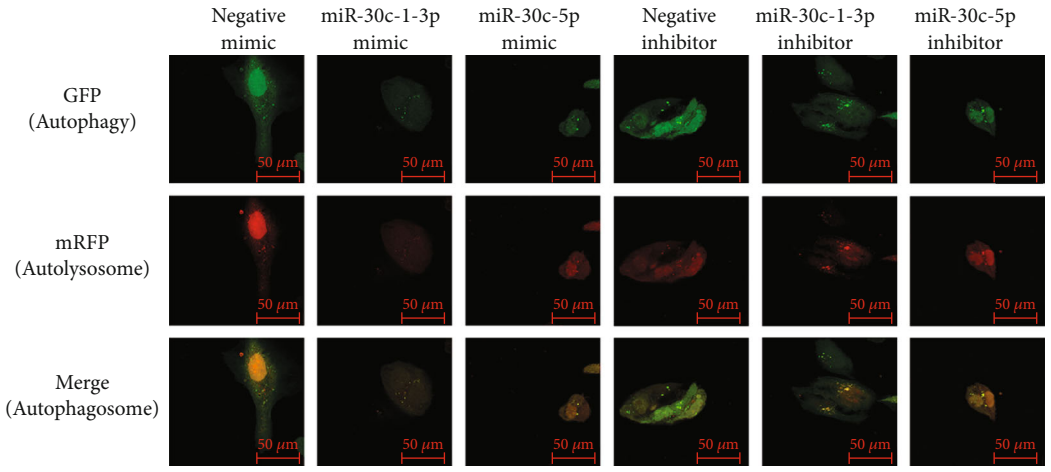


(a)

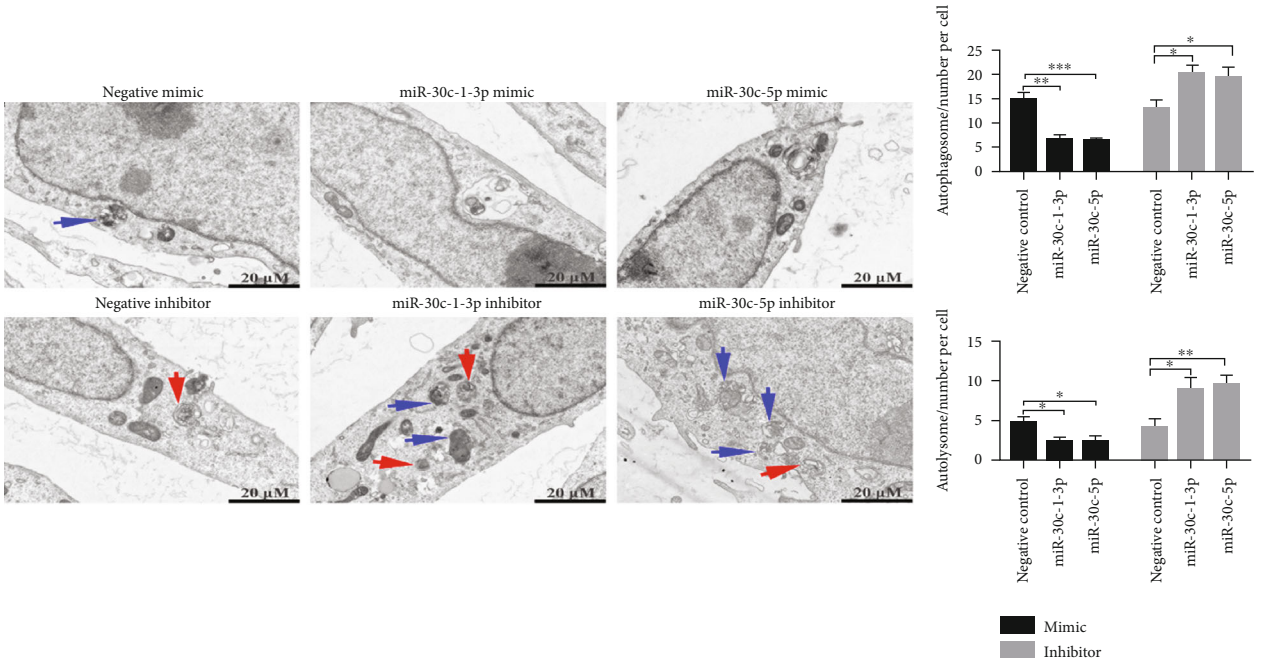


(b)

Figure 3: Continued.



(c)



(d)

FIGURE 3: The expression of miR-30c-1-3p and miR-30c-5p is upregulated in response to *H. pylori* infection and downregulates xenophagy. (a) Quantitative real-time PCR detection of miR-30c-1-3p and miR-30c-5p after infection. In GES-1 cell lines, the expression of miR-30c-1-3p and miR-30c-5p rapidly increased after being infected with *H. pylori* ($n = 5$). (b) GES-1 cells were transfected with mimic (100 nM) and inhibitor (100 nM) of miR-30c-1-3p as well as miR-30c-5p, negative mimic (100 nM) or negative inhibitor (100 nM) for 36 h, afterwards infected with *H. pylori* for 12 h. The expressions of LC3B-II and P62 were measured by western blot. (c) Quantification of autophagosomes and autolysosomes. GES-1 cells were transfected with mimic (100 nM) and inhibitor (100 nM) of miR-30c-1-3p as well as miR-30c-5p, negative mimic (100 nM) or negative inhibitor (100 nM) for 24 h. Afterwards, mRFP-GFP-LC3 adenovirus was transfected for 24 h and finally stimulated with *H. pylori* for 12 h. The number of spots per cell was counted ($n = 5$). (d) *H. pylori* infected GES-1 cells for 12 h after transfection with mimic (100 nM) and inhibitor (100 nM) of miR-30c-1-3p as well as miR-30c-5p, negative mimic (100 nM) or negative inhibitor (100 nM) for 36 h. The samples were collected for TEM examination to observe ultrastructural alterations. Red arrows indicate autophagosomes, and blue arrows indicate autolysosomes. All error bars indicate SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Xenophagy can protect the body from infection by various pathogens and play an important role in maintaining body homeostasis [24]. *H. pylori* can induce xenophagy in multiple cell lines [11, 25]. In this study, we showed that *H. pylori* infection induced LC3B-II and P62 accumulation in GES-1 cell lines. The underlying reasons for this phenomenon may be that the promotion of autophagy flux in the initial stage and the inhibition of the degradation stage are induced by *H. pylori*. Furthermore, we use a selective inhibitor of autophagy flux degradation stage, bafilomycin A1 (Baf A1) to verify the hypothesis. We confirmed that *H. pylori* infection induced cellular autophagy flux but inhibited the

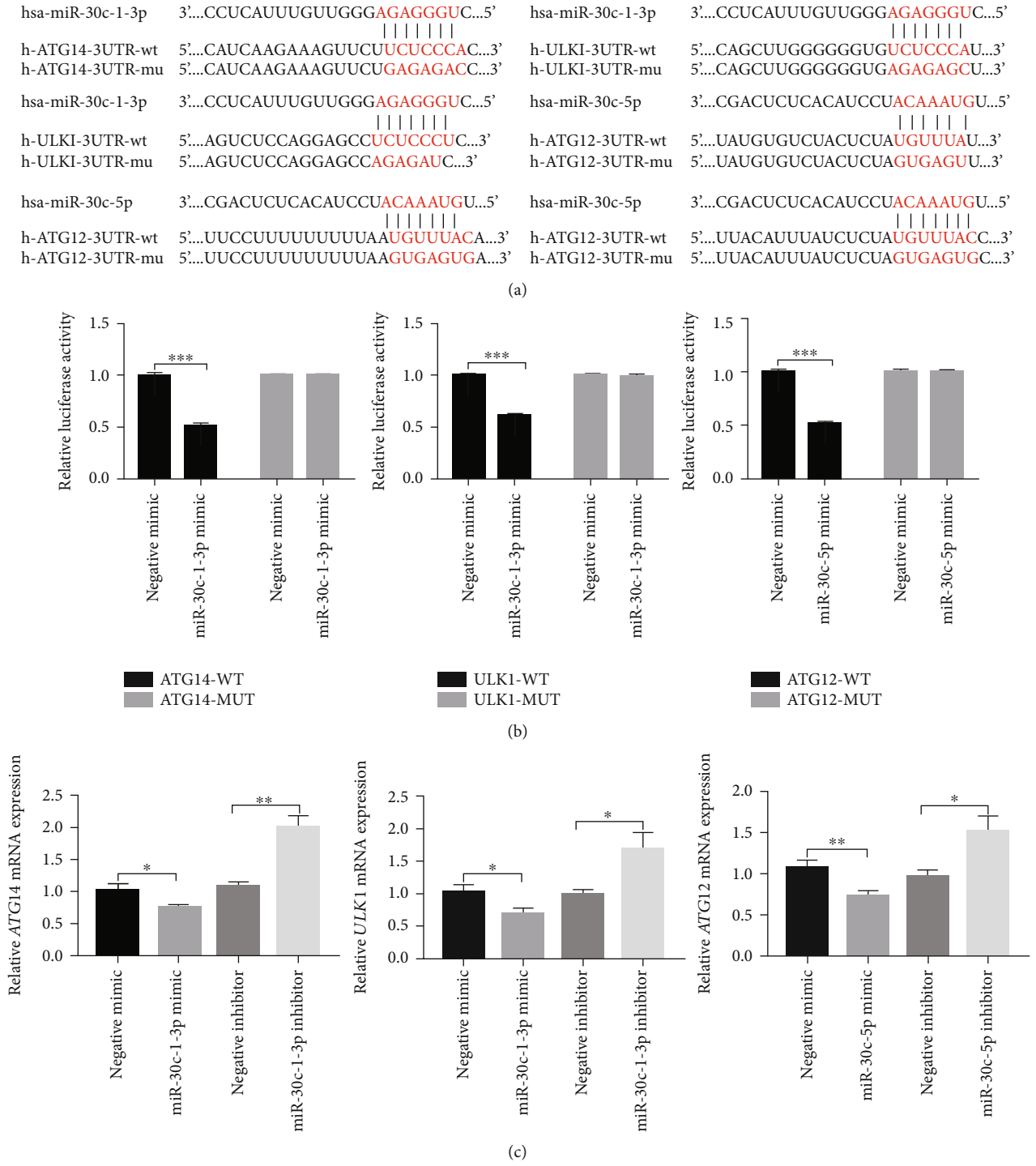


Figure 4: miR-30c-1-3p and miR-30c-5p regulated target autophagy-related genes at mRNA level through mRNA degradation. (a) Underlying target site for miR-30c-1-3p on the 3′UTR of *ATG14* and *ULK1* mRNA and underlying target site for miR-30c-5p on the 3′ UTR of *ATG12* mRNA were forecast by TargetScan7.2 and miRDB. (b) Dual-luciferase reporter assay was used to confirm the interactions between miR-30c-1-3p and 3′UTR of *ATG14* and *ULK1*, as well as miR-30c-5p and 3′UTR of *ATG12*. (c) GES-1 cells were transfected with mimic (100 nM) and inhibitor (100 nM) of miR-30c-1-3p as well as miR-30c-5p, negative mimic (100 nM) or negative inhibitor (100 nM) for 36 h. The mRNA levels of *ATG14*, *ULK1* and *ATG12* were detected by RT-qPCR ($n = 6$). All error bars indicate SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

degradation stage. Several published studies have revealed that VacA and CagA can destroy autophagy flux and inhibit the digestive function of lysosomes [26, 27]. The latest research also confirms that *H. pylori* inhibits autophagy flux by downregulating SIRT1 and promotes its intracellular survival and colonization [28]. Given the complexity of *H. pylori*

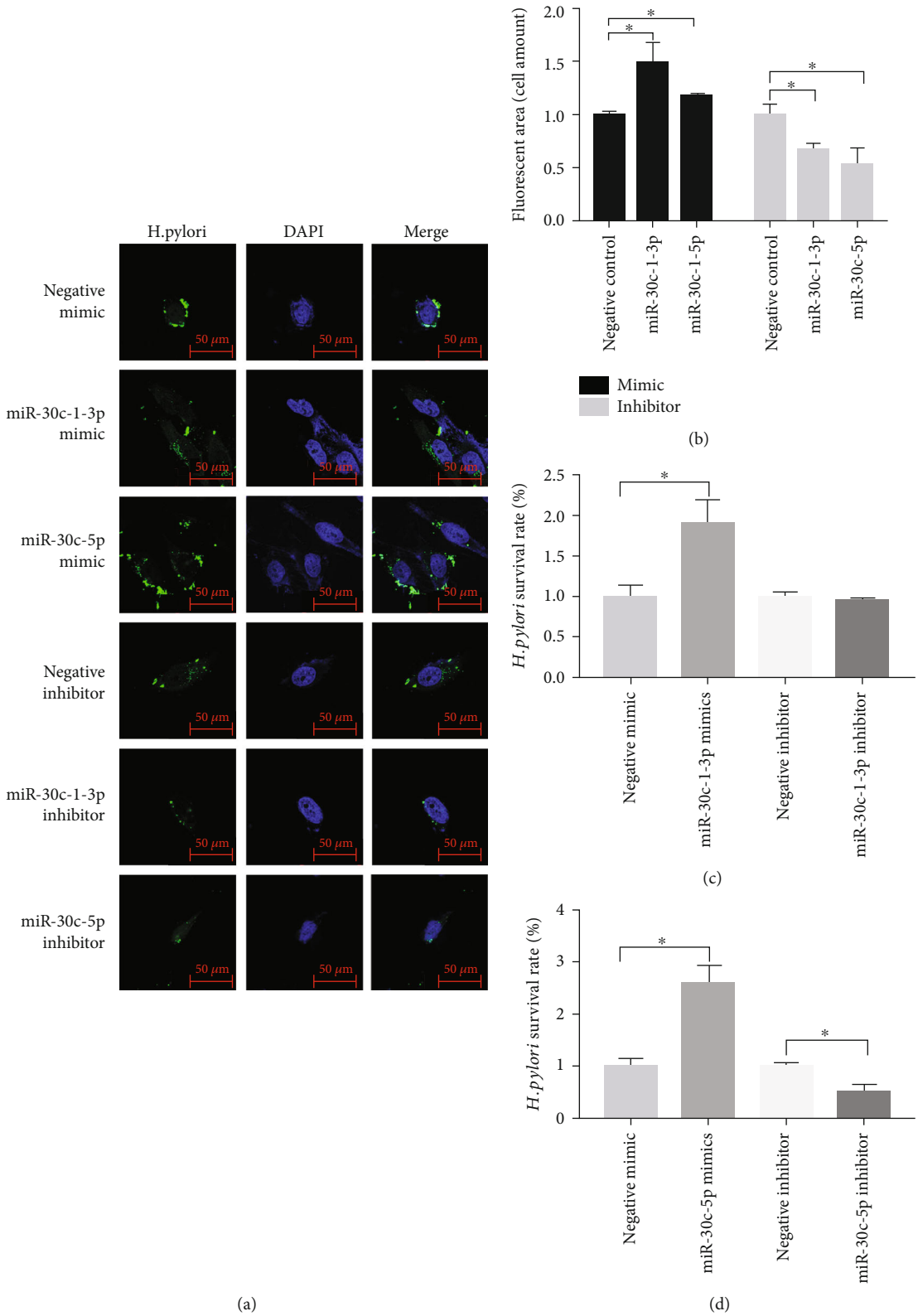


Figure 5: miR-30c-1-3p and miR-30c-5p decrease the intracellular *H. pylori* elimination in GES-1 cells. (a, b) After GES-1 cells were transfected with mimic and inhibitor of miR-30c-1-3p as well as miR-30c-5p, negative mimic or negative inhibitor for 36 h, and then stimulated with *H. pylori* for 12 h. Intracellular survival of *H. pylori* was detected by gentamicin protection assay. (c, d) After GES-1 cells were transfected with mimic and inhibitor of miR-30c-1-3p as well as miR-30c-5p, negative mimic or negative inhibitor for 36 h, and then stimulated with *H. pylori* for 12 h. The extracellular *H. pylori* were eliminated with gentamicin. The cells were dyeing with anti-*H. pylori* antibodies (green) and DAPI (blue). All error bars indicate SEM. $^{*}P < 0.05$.

infection, synergistic effect induced by many factors may be involved in xenophagy inhibition.

H. pylori can also inhibit xenophagy through miRNA-related mechanisms [29]. For example, it can regulate xenophagy through the miR-30 family. Members of the miR-30 family can bind to the 3′-UTR regions of BECN1 mRNA, thereby inhibiting the expression of BECN1 mRNA and finally inhibiting xenophagy [30]. The same miRNA may target different pathway, and different miRNAs may regulate the same target [31]. In our study, we found that *H. pylori* infection increased the expression of miR-30c-1-3p and miR-30c-5p in GES-1 cell lines. Besides, miR-30c-1-3p and miR-30c-5p played a negative regulatory role in xenophagy induced by *H. pylori* infection in GES-1 cell lines. However, studies have reported that miR-30c-5p relieves kidney stones by targeting ATG5 in human renal tubular epithelial cells [32]. More researches are needed to confirm the regulatory roles of miR-30c-1-3p and miR-30c-5p in different cell lines, tissues, organs, and even different disease processes. The intracellular survival and proliferation of *H. pylori* are one of the key factors of persistent infection in drug resistance. Resistance of *H. pylori* to a variety of antibiotics is an urgent problem to be solved, whether miR-30c-1-3p and miR-30c-5p are involved in the regulation of drug resistance and the regulatory mechanism need to be further studied. Furthermore, using animal models to evaluate the effects of miRNA on intracellular *H. pylori* is a technical threshold that we are trying to achieve.

H. pylori causes epigenetic changes by affecting miRNA expression, it can also be causes of epigenetic changes by affecting DNA methylation and histone modification. *H. pylori* infection induces aberrant methylation in a number of gene promoters in gastric mucosa. In mouse macrophages, *H. pylori* induces H3S10 phosphorylation at the *IL-6* promoter, which plays an important role in increased IL-6 mRNA and protein expression. Direct exposure of *H. pylori* to gastric epithelial cells causes upregulation of $p21^{WAF1}$ protein expression, which associated with increased HDAC1 recruitment from the $p21^{WAF1}$ promoter and hyperacetylation of histone H4 [33].

Studies have shown that *ATG14*, *ULK1*, and *ATG12* play an important role in the formation of autophagosome [23]. In our research, we confirmed that miR-30c-1-3p and miR-30c-5p reduce the expression of multiple key autophagy genes at mRNA level. Whether miR-30c-1-3p and miR-30c-5p can affect the expression of target genes at the protein level needs further verification. Studies have shown that miR-30b-5p inhibits the transcription factor EB- (TFEB-) dependent transactivation by binding to the coordinated lysosomal expression and regulation (CLEAR) elements in the nucleus to regulate the lysosomal biogenesis and autophagy [34]. As another member of the miR-30 family, it is not clear whether miR-30c-1-3p and miR-30c-5p can regulate the lysosomal biogenesis and autophagy through the TFEB-dependent transactivation in the nucleus during *H. pylori* infection.

On the basis of our experimental results, we concluded that the repression of xenophagy by miR-30c-1-3p and miR-30c-5p by targeting *ATG14*, *ULK1*, and *ATG12* benefitted intracellular *H. pylori* evade xenophagy clearance. Although the cause of persistent *H. pylori* infection has not been fully confirmed, this study established a foundation that is necessary for the future evaluation of the role of miR-30c-1-3p and miR-30c-5p in *H. pylori* infections. Thus, these findings provide a novel molecular mechanism for persistent *H. pylori* infection and a new target against *H. pylori*.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Additional Points

Take Aways. (i) *H. pylori* infection blocked the degradation stage of autophagy flux in GES-1 cell line. (ii) Inhibition of xenophagy helps *H. pylori* survive in cells. (iii) miR-30c-1-3p and miR-30c-5p played a negative regulatory role in xenophagy induced by *H. pylori* infection. (iv) miR-30c-1-3p and miR-30c-5p reduce the expression of multiple key autophagy-related genes at mRNA level. (v) miR-30c-1-3p and miR-30c-5p decrease intracellular *H. pylori* elimination in GES-1 cells.

Conflicts of Interest

All authors declare that there is no conflict of interest and had final approval of the submitted and published versions.

Authors' Contributions

Qiuhua Deng, Yifei Xu, Si Du, Jiongming Yang, and Lingping Wu performed the experiments. Qiuhua Deng, Yifei Xu, and Yuanzun Zhong wrote manuscript. Liyao Tang, Yuanzun Zhong, and Shaoju Guo summarized and analyzed the data. Yifei Xu and Bin Huang revised the manuscript. Hongying Cao and Ping Huang designed the study and finally revised the manuscript. Qiuhua Deng and Yifei Xu contributed equally to the work.

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Supplementary Materials

Supplementary materials contain the original datas from this study. *(Supplementary Materials)*

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