

Original Article

Effect of *Helicobacter pylori* on the expression of semaphorin 5A in patients with gastric precancerous lesions and its clinical significance

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Abstract: Semaphorin 5A, a member of semaphorin family, was originally identified as functional axonal guidance factor during neuronal development. Previously, we showed that the expression of semaphorin 5A might contribute to the occurrence and development of gastric cancer. However, it is unclear whether semaphorin 5A is involved in *H. pylori*-related gastric precancerous lesions. In this study, a total of 120 patients of gastric precancerous lesions, of which 60 were *Helicobacter. pylori* (*H. pylori*) positive (observation group) and other 60 were negative controls, were selected. The expression of semaphorin 5A mRNA and protein in the gastric precancerous lesion tissues was analyzed by RT-qPCR and immunohistochemistry, respectively. In addition, *in vitro* experiments were performed to investigate the effect of *H. Pylori* infection on semaphorin 5A expression in normal human gastric mucosal GES-1 cells. Compared with control group, the mRNA and protein expression levels of semaphorin 5A in observed group were significantly increased ($P<0.05$). *H. pylori* co-culture upregulated semaphorin 5A gene expression in GES-1 cells. We conclude that semaphorin 5A gene expression may be a major determinant in the occurrence and development of *H. pylori*-related gastric precancerous lesions, which could be a novel potential molecular biomarker for prevention and early detection of gastric cancer.

Keywords: Semaphorin 5A, gastric precancerous lesion, *Helicobacter pylori* (*H. pylori*)

Introduction

Gastric cancer is the third leading global cause of cancer-related death, ranked as the fifth most common malignant tumor [1]. It has been proposed that the gastric cancer development is a dynamic process of a precancerous cascade involving with lesions, such as atrophic gastritis, intestinal metaplasia and dysplasia [2]. Persistent *H. pylori* infection induces gastric chronic inflammation, resulting in a transition from normal mucosa to chronic atrophic gastritis followed by intestinal metaplasia and dysplasia, and subsequently increases the risk of gastric cancer [3, 4]. However, the mechanism underlying the *H. pylori*-induced occurrence and development of gastric precancerous lesions remains to be fully elucidated.

Semaphorin 5A belongs to class 5 of the semaphorin family, and is an integral membrane protein with seven characteristic thrombospondin specific repeats (TSP-1), which was originally identified as functional axonal guidance factor during neuronal development [5]. Our previous studies have demonstrated that semaphorin 5A is overexpressed in gastric cancer tissue, which may be closely associated with tumorigenesis and metastasis of gastric cancer [6-9]. However, it is unclear whether semaphorin 5A is involved in *H. pylori*-related gastric precancerous lesions. The aim of the present study was to analyze the effect of *H. pylori* infection on the expression level of semaphorin 5A in gastric precancerous lesion tissues and in normal human gastric mucosal GES-1 cells *in vitro*, and to explore the clinical significance of semapho-

rin 5A in the development of *H. pylori*-induced gastric precancerous diseases.

Materials and methods

Human gastric tissue specimens

Human gastric mucosa disease specimens were randomly obtained from 120 patients, who were diagnosed with gastric precancerous lesions by gastroscopy and pathological examination in the First Affiliated Hospital, Kunming Medical University (Kunming, China) between August 2014 and July 2015. Pathological diagnosis was classified according to the diagnostic criteria formulated by the National Chronic Gastritis Conference. Of patients, 60 were confirmed to be *H. Pylori*-positive by Giemsa staining and the rapid urease test, and they were classified as the observation group; the other 60 patients were *H. Pylori*-negative and referred to as the control group. The observation group comprised 24 cases of chronic atrophic gastritis, 22 cases of the intestinal metaplasia of gastric mucosa and 14 cases of the atypical hyperplasia of gastric mucosa (aged from 31-67 years, median at 48 years; 34 male and 26 female patients). The control group included 22 cases of chronic atrophic gastritis, 21 cases of the intestinal metaplasia of gastric mucosa and 17 cases of the atypical hyperplasia of gastric mucosal (aged from 39-73 years, median at 50 years; 32 male and 28 female patients). The obtained tissues were immediately frozen at liquid nitrogen and stored in -70°C for the later extraction of total RNA. The acquisition of tissue specimens and study protocol were approved by the Kunming Medical University Institutional Review Board, and was performed in strict accordance with the regulations. Written informed consents were obtained from all of the patient subjects.

Reverse transcription real-time quantitation polymerase chain reaction (RT-qPCR)

Total RNAs from biopsy tissue or *H. Pylori*-treated cells were prepared with Trizol (Invitrogen) according to the manufacturer's protocol under RNase-free condition. After cDNA was synthesized by using a two-step reverse transcription reaction kit (Invitrogen), quantitative PCR was performed on an Applied Biosystems 7500 Real-time PCR System by using SYBR Premix Ex Taq kit (Applied Biosystems,

USA) in Axygen 96-well reaction plates, following the manufacturer's instructions. β -actin was amplified to serve as a reference for each sample and the observed semaphorin 5A expression level was normalized to the level of β -actin. PCR conditions were as follows: an initial melting step at 95°C for 1 min followed by 35 cycles at 95°C for 90 s, at 60°C for 30 s, at 72°C for 30 s, and a final extension at 72°C for 10 min. The primers for were forward 5'-GGT ACT GTT CTA GCG ACG GC-3' and reverse 5'-ATA CTT GGG TTC GGG GTT GT-3' for semaphorin 5A; and forward 5'-TGA CGT GGA CAT CCG CAA AG-3', and reverse 5'-CTG GAA GGT GGA CAG CGA GG-3' for β -actin, respectively.

Western blotting analysis

The cells were homogenized in lysis buffer. Equal amounts of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% nonfat milk and incubated with primary antibody (Ab). After washed 3 time (15 min each) with tris-buffered saline (TBS) supplemented with 0.1% Tween 20 (TBST), the membrane was incubated with horseradish peroxidase conjugated rabbit anti-mouse secondary Ab, followed by enhanced chemi-luminescence (KPL, Gaithersburg, USA). Primary antibodies against semaphorin 5A, β -actin were applied at the optimized concentrations (Santa Cruz Biotechnology).

Immunohistochemistry (IHC) analysis

Formalin-fixed, paraffin-embedded tissue samples were cut into 4 μ m sections and mounted on adhesion microscope slides. Immunohistochemical (IHC) staining for semaphorin 5A was performed by automation using a Ventana BenchMark ULTRA instrument with mouse polyclonal anti-semaphorin 5A antibody. The expression level was determined by scoring both the positivity percentage and intensity of semaphorin 5A staining. The positive expression percentage was determined by counting the number of immunopositive cells in three areas randomly chosen in the tissue with a total of 300 cells and scored (0=0%, 1=1%~25%, 2=26%~35%, 3=36%~45%, 4=46%~65%, 5=66%~100%). The staining intensity was scored as follows: 0, no detectable cell membrane and cytoplasmic staining; 1, mild

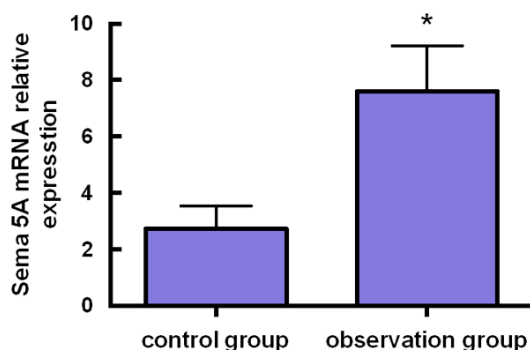


Figure 1. Semaphorin 5A mRNA expression in the gastric mucosa lesions of observation group vs control group. * $P < 0.05$.

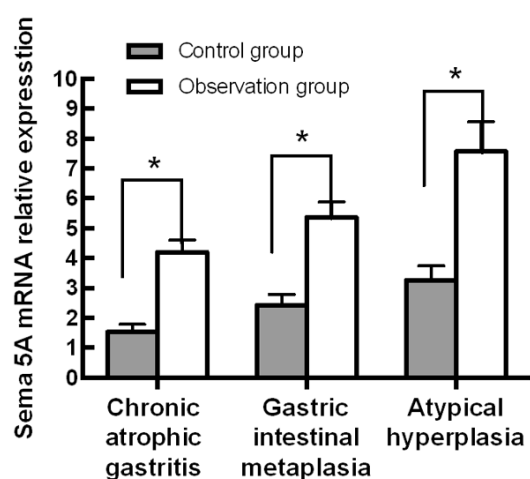


Figure 2. Semaphorin 5A mRNA expression in different types of pathological gastric mucosa lesions for observed group vs. control group: chronic atrophic gastritis, gastric intestinal metaplasia, and atypical hyperplasia. * $P < 0.05$.

cell membrane and cytoplasmic staining; 2, moderate cell membrane and cytoplasmic staining; and 3, severe cell membrane and cytoplasmic staining. The two scores were then summed to obtain a final score, represented 0, +, ++, +++.

Cell infection experiment in vitro

Normal human gastric mucosal GES-1 cells were cultured to the exponential growth phase in DMEM (GIBCO, Carlsbad, CA, USA) containing 10% heat inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 Ig/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, and then digested with 0.25% pancreatin. The cell concentra-

tion was adjusted to 4×10^5 /ml, and the cells were inoculated in a 6-well plate with 2 ml per hole. Following 12 h of culture, when the cells were completely attached to the wall. The H. Pylori densities were adjusted by optical density (OD) measurement at 660 nm, in which OD₆₆₀ = 1×10^8 colony-forming unit (CFU)/ml. H. Pylori was then incubated with GES-1 cells at a cell-to-bacterium ratio of 1:50 or 1:100 for up to 12 h or 24 h in the medium.

Statistical analysis

All experiments were performed at least three times, and the results from the representative experiments were used for the statistical analysis by using software SPSS 13.0. The measurement data were presented as mean $\pm$ SD. Clinical pathological findings were compared using an unpaired t-test or Pearson χ^2 test. The statistical analysis of the data between the control and treated groups was performed using analysis of variance (ANOVA). A value of $P < 0.05$ was considered a statistically significant.

Results

Semaphorin 5A mRNA expression in the gastric mucosa of the two groups

To check the effect of H. Pylori on occurrence and development of gastric precancerous lesions, we first measured the mRNA expression of semaphorin 5A in the gastric mucosa lesions using RT-qPCR method. As shown in **Figure 1**, the semaphorin 5A mRNA expression in the gastric mucosa of the patients in the observation group is significantly upregulated compared to that of control group ($P < 0.05$). **Figure 2** shows the representative qPCR results of semaphorin 5A mRNA expression for different types of pathological lesions. The values of observed group are significantly higher than those of the control group ($P < 0.05$).

Semaphorin 5A protein expression in the gastric mucosa of the two groups

Semaphorin 5A protein expression in the gastric mucosa lesions was measured by IHC. A weak to moderate positive reactivity of semaphorin 5A was found in the gastric mucosa of the control patient group. However, semaphorin 5A protein expression in the gastric mucosa of

Table 1. Semaphorin 5A expression is associated with *H. Pylori* infection

Variable	Semaphorin 5A expression				Total	χ^2	p
	0	+	++	+++			
The observation group	3	11	21	25	60	12.38	<0.001
The control group	11	43	6	0	60		

the observation group was quite visible and significantly higher than that in the control group as shown in **Table 1** ($P<0.01$). **Figure 3** shows the semaphorin 5A protein levels in the gastric mucosa of different lesions in the observed group with the values significantly higher than those in the control group ($P<0.05$), which is consistent with the mRNA results in RT-qPCR analysis.

Effect of H. Pylori infection on semaphorin 5A expression in gastric mucosa cell

To investigate whether semaphorin 5A is involved in *H. pylori*-related gastric precancerous lesions, we performed in vitro experiments. *H. Pylori* was used to infect gastric mucosa GES-1 cell line. The results shows that *H. Pylori* infection leads a upregulation of semaphorin 5A protein in GES-1, which is of dose- and time-dependent. As shown in **Figure 4A**, semaphorin 5A expression increases by 2.7 fold at 12 h and over 4-fold at 24 h after treated with *H. Pylori* (1:50) in GES-1 cells vs. those of the untreated group (0 h). **Figure 5A** shows a higher expression of semaphorin 5A protein in GES-1 cells co-cultured with *H. Pylori* in a ratio of cells to bacteria ranged from 1:50 to 1:100, which is about 1.5 to 3-fold higher than those of the untreated cells (1:0).

To further confirm the effect of *H. Pylori* infection on semaphorin 5A expression, RT-qPCR assay was performed to examine the mRNA transcript level of semaphorin 5A in GES-1 cells after co-culture with *H. pylori*. We found that *H. Pylori* were significantly upregulated the mRNA transcript level of semaphorin 5A in GES-1 cells ($P<0.05$), and the upregulation change is of dose- and time-dependent (**Figures 4B, 5B**).

Discussion

The association between precancerous lesions and adenocarcinoma of the stomach is well known. The formation of precancerous lesions

is an important stage in the transformation process from normal gastric mucosa to gastric carcinoma, initiating as chronic gastritis followed by atrophy, intestinal metaplasia and dysplasia. *H. Pylori* infection causes a series of precancerous lesions like gastritis, atrophy, intestinal metaplasia and dysplasia, and is the strongest

known risk factor for gastric cancer, as supported by epidemiological, preclinical and clinical studies [10]. Therefore, it is of great clinical value to further study mechanism underlying the *H. pylori*-induced occurrence and development of gastric precancerous lesions and find novel therapeutic strategies that specifically suppress the process.

Semaphorin 5A, a member of class-5 semaphorins, is a transmembrane protein that is located in chromosome 5p15.2 in human [11]. Initially, the gene was identified as constituents of the complex regulatory system responsible for the guidance of growing axons to their targets during the development of the central nervous system [5]. However, cumulative data have indicated that certain semaphorins implicated in axonal path finding in the developing nervous system were found to be expressed in multiple types of cancer cells, to modulate the behavior of cancer cells and to promote tumor angiogenesis and tumor progression by multiple mechanisms [12-15]. In our previous study, we have observed and described the possible contribution of smaphorin 5A in the development and progression of gastric carcinoma [6-9]. However, we need to have a better understanding of how semaphoring 5A is involved in a detail mechanism, and it is unclear whether semaphorin 5A is involved in *H. Pylori* infection-related gastric disease. There are no data available about the expression of semaphorin 5A in *H. Pylori* infection-related gastric mucosa lesions. In our previous preliminary studies, we speculated that semaphorin 5A might be a critical molecular determinant in the occurrence and development of *H. Pylori*-related gastric diseases.

In the present study, the mRNA data detected by using RT-qPCR indicate that the semaphorin 5A is increased significantly in the patients with *H. Pylori*-positive gastric precancerous lesions vs. the control patients with *H. Pylori*-negative

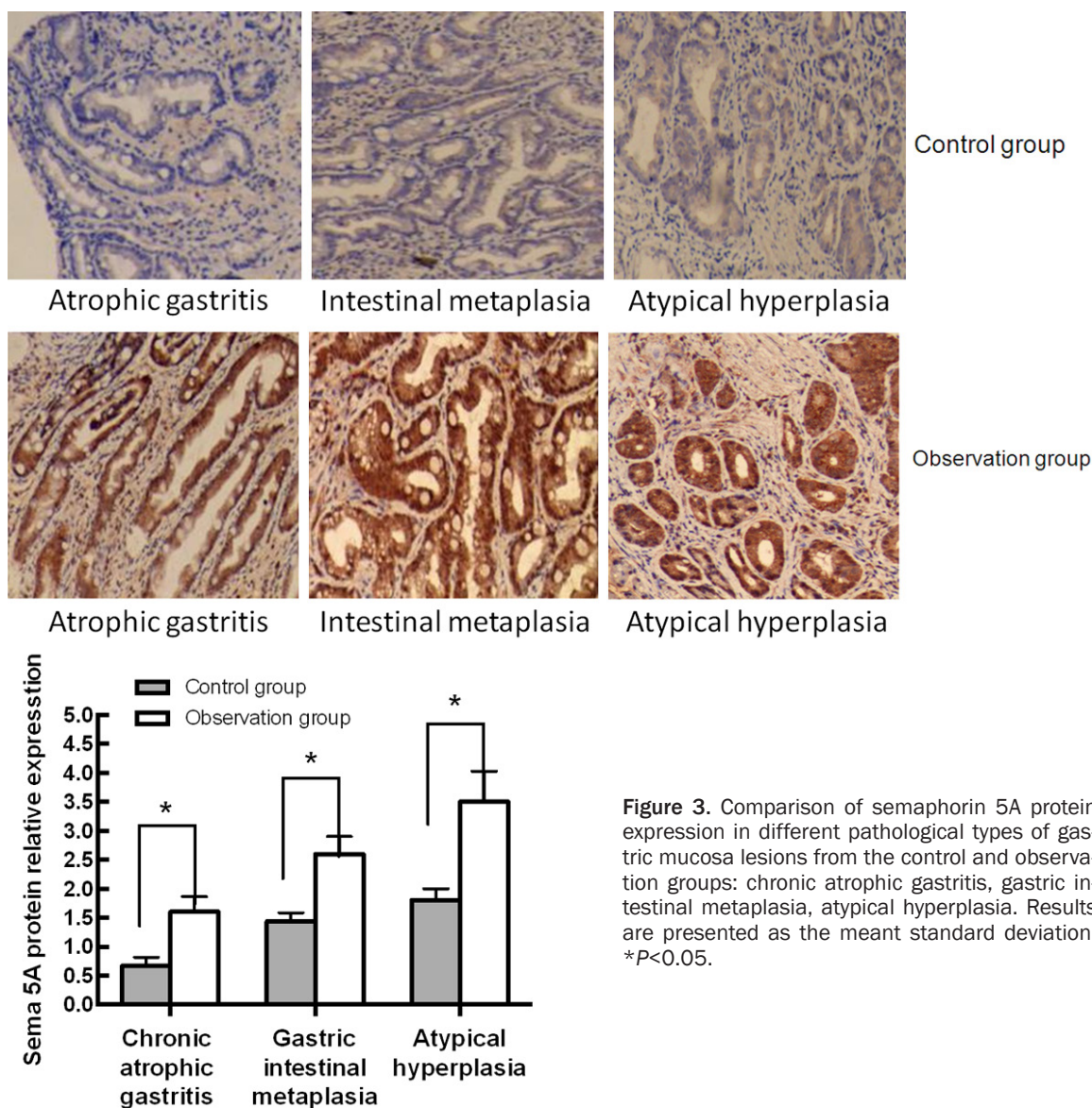


Figure 3. Comparison of semaphorin 5A protein expression in different pathological types of gastric mucosa lesions from the control and observation groups: chronic atrophic gastritis, gastric intestinal metaplasia, atypical hyperplasia. Results are presented as the mean standard deviation. * $P<0.05$.

lesions. mRNA measurements in different types of pathological gastric mucosa lesions support this observation. This tendency is further confirmed in our IHC experiments of gastric precancerous lesions. Our *in vitro* experiments on normal gastric mucosa GES-1 cells provide additional evidences supporting our previous speculation that semaphorin 5A is the key in *H. Pylori* infection-related gastric precancerous lesions.

Taken together, the present study suggested that *H. Pylori* infection may stimulate the gastric mucosa, resulting in an abnormal activation of semaphorin 5A transcription and translation, and that *H. Pylori* infection may induce

the occurrence and development of gastric precancerous lesions through semaphorin 5A expression.

In conclusion, semaphorin 5A mRNA and protein are expressed significantly higher in the gastric mucosa of patients with *H. Pylori* infected precancerous lesions. This gene upregulation is also observed in *H. Pylori* infected normal gastric GES-1 cells. The results indicate that semaphorin 5A is involved in the formation of *H. Pylori* infection-related precancerous lesions, an important stage in the transformation process from normal gastric mucosa to gastric carcinoma. Our previous studies observed the correlation between semaphorin 5A

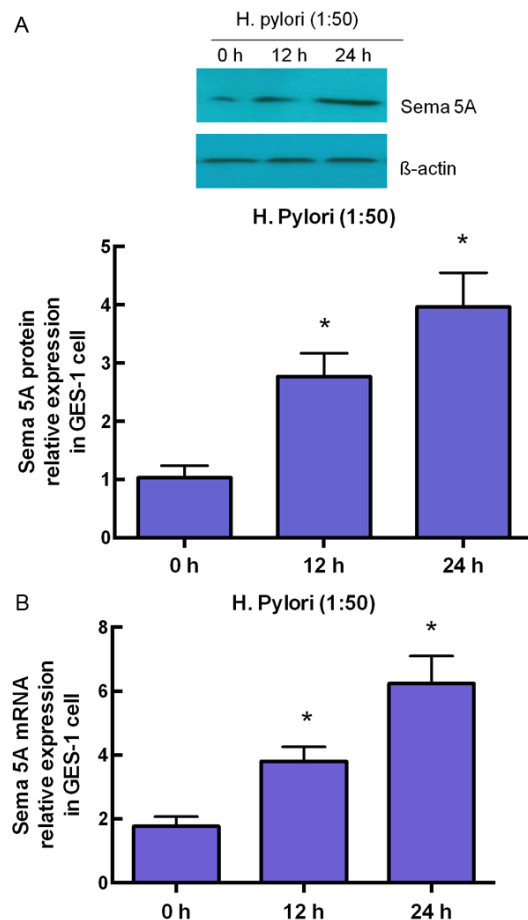


Figure 4. *H. pylori* treatment upregulated semaphorin 5A expression in normal human gastric mucosal GES-1 cell in vitro. Cultured GES-1 cell was treated with *H. pylori* (1:50), the expression of semaphorin 5A protein and mRNA was analyzed by Western blot and RT-qPCR at different time points (0 h, 12 h, 24 h) (A, B). Results are presented as the mean standard deviation. * $P < 0.05$, compared with untreated (0 h) group.

expression and the pathogenesis of the gastric cancer. The present findings provide further information to understand the mechanism of *H. Pylori* carcinogenesis and a potential strategy for the treatment of *H. Pylori*-associated gastric carcinoma. Our keystone of this study is that semaphorin 5A could be a novel molecular biomarker for prevention and early detection of gastric cancer.

Disclosure of conflict of interest

None.

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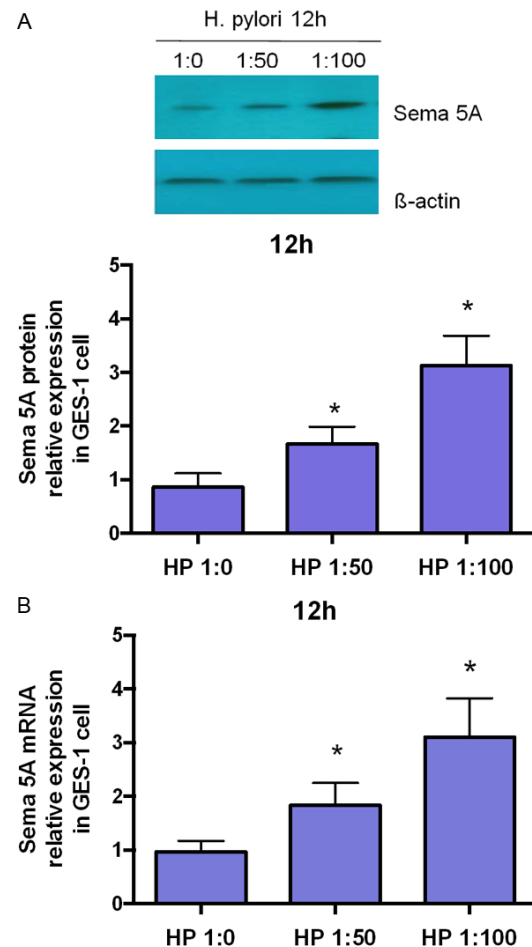


Figure 5. *H. pylori* treatment upregulated semaphorin 5A expression in normal human gastric mucosal GES-1 cell in vitro. The cell was treated with various doses of *H. pylori* (1:0, 1:50, 1:100), the expression of semaphorin 5A protein and mRNA was analyzed by Western blot and RT-qPCR after 12 h (A, B). Results are presented as the mean standard deviation. * $P < 0.05$, compared with untreated (1:0) group.

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